Food Research

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Flavor in Foods—A Cumulative Review

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INSIDE SCIENCE

The Vital Story of VITAMIN C

by Science Writer

What does vitamin C do for human beings?

Medical research shows that it is essential in maintaining healthy bony tissue and for the formation of collagen which is the main supportive protein of skin, tendon, bone, cartilage and connective tissue. It is needed for vascular function and tissue respiration and in promoting the healing of wounds. In lactating women it has great importance in providing the infant with a sufficient amount of ascorbic acid to prevent scurvy, a disease to which very young babies are susceptible.

Much evidence is available in medical reports to show that a lack of this vital nutritive element can result in impaired health. And in those cases of frank scurvy which still occur, ascorbic acid is the specific medication.

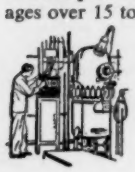
History. The history of vitamin C is fascinating. Scurvy was once the most prevalent disease in Europe. In the 18th Century the antiscorbutic value of fresh vegetables and citrus fruits was recognized.

Isolation. It was not until 1918-25 that almost pure ascorbic acid was concentrated from the lemon by Zilva and its important properties and molecular composition established. In 1927 he concluded that this so-called "reducing factor" and the antiscorbutic principle were closely related. In 1928, Szent-Györgyi, who started investigations in 1921, isolated a strong reducing compound from adrenal glands, oranges, cabbage, terming it "hexuronic acid."

Chemistry. The "reducing factor," "hexuronic acid," and vitamin C were all identified as the same in 1932 by various groups of workers. This was one year after C. G. King established the chemical identification of vitamin C.

Synthesis. In 1933 Reichstein and his colleagues in Switzerland successfully synthesized ascorbic acid. Almost simultaneously in England Haworth and associates accomplished the same. The Reichstein synthesis formed the basis for large scale commercial production by the world-famous firm of Hoffmann-La Roche.

Production. Following these discoveries came the establishment of manufacturing operations so that today daily production by the tons is the rule. The figures are really staggering. The amount produced weekly in the U. S. A. averages over 15 tons, equivalent to that contained in over 200 million oranges. It is being made synthetically at a very low cost which permits widespread use in many food and drug products. One kilo is sufficient to supply 92 people for one year with the minimum daily requirements set by the U. S. Food and Drug Administration.



Vitamin C made by the Roche process is identical chemically and in biological activity with Nature's own product. Because vitamins can be manufactured at a much lower cost than if they were extracted from natural sources they are widely used in dietary supplements.

Ascorbic acid is present in many foods. Nature supplies some with lavish amounts and stints on others. In fact, wide variations* have been found in the vitamin C content in varieties of the same fruit.

Deficiencies. Many surveys have shown widespread vitamin C deficiencies in the population. Physicians may call these subclinical, but they are deficiencies nevertheless.

Because of these situations—natural variation and widespread deficiencies—diet experts believe that it is desirable and in the public interest to standardize the vitamin C content of processed fruit and vegetable juices by the addition of enough pure crystalline ascorbic acid to make them consistently dependable sources of this necessary food element. Some of these juices are: tomato, grape, orange, grapefruit, pineapple.

Requirements. The U. S. Food & Drug Administration has set 30 milligrams as the adult minimum daily requirement of ascorbic acid. This daily intake is just about sufficient to prevent recognizable signs of vitamin C deficiencies although a subclinical or an unrecognized deficiency may exist. In view of this, the National Research Council has established 75 milligrams as the recommended daily male adult allowance and 70 mg. daily for female adults, which levels are supported by many controlled nutrition studies. However, the N. R. C. recommends 90 mg. daily for boys between 13 and 15, 100 mg. daily for boys between 16 and 20, and 150 mg. daily for lactating women.

This article is published in the interests of pharmaceutical manufacturers and food processors who make their good products better with essential, health-giving vitamin C. Reprints are freely available. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon Street, St. Laurent, P. Q.

*The table shows minimum and maximum levels of ascorbic acid in commercially canned juices. All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	*Max.*
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Tomato juice	2.5	32.0

AN EXAMINATION OF THREE METHODS FOR TESTING PALATABILITY AS ILLUSTRATED BY STRAWBERRY FLAVOR DIFFERENCES[a]

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(Manuscript received August 17, 1956)

Currently, there is widespread use of sensory testing as a laboratory tool for assessing food quality. Taste tests are employed to determine the effects of additives, pesticides, varietal differences, processing methods, storage, and other influences on the flavor, texture and color of foods. Frequently, they are less time-consuming than chemical or physical tests of quality *(3)*. In 1950, the primary conclusion resulting from a conference *(9)* on sensory testing was that more experimental investigations were needed on methodology. After 7 years and many methodology studies that recommendation still holds, though important advances have been made.

Many investigators involved in quality testing are prone to use only the method which is familiar and easiest under their circumstances. Oftentimes this approach is justifiable, at least from a practical viewpoint. The investigator is under pressure to evaluate test material. There is no time to accumulate data strictly limited to methodology. Thus, expediency determines the pattern.

In this laboratory we have used the ranking technique almost to the exclusion of other methods, primarily because it may be used effectively with untrained judges.

Occasionally we have compared the same samples by 2 methods, e.g. ranking vs. scoring, or ranking vs. paired comparisons. In these instances the arrays of means have been sufficiently alike to lead us to believe that the results justify a continued use of the ranking technique. We have not heretofore probed too deeply into minor differences, because we were unaware of any statistical technique to demonstrate real differences attributable to method.

In the summer of 1955 an experiment was designed to compare methods, using fresh strawberries as the test material. The test was limited to 3 varieties in order to economize on the time involved in serving and analyzing results and on the supply of sample material.

On the basis of a previous study *(14)* by a ranking method made on 6 varieties of frozen strawberries, we knew that the flavor of the variety Sparkle was highly preferred to that of the Monmouth variety. We further knew that the flavor of the variety Orland was at a low preference level. Therefore, those 3 varieties were chosen for our test material. We did *not* know, of course, whether the flavor differences among those varieties were intrinsic in

[a] Some of the data reported herein were presented at a joint meeting of the Biometrics Society and the American Statistical Association in a session on Subjective Testing, 29 Dec. 1955. Biltmore Hotel, New York, N. Y.

the fresh berries or could be attributed to the freezing. The only evidence available on the quality of the 3 varieties in the fresh state was the opinion of growers and that of various people at the University who are concerned with strawberries. However, we felt justified in assuming that true flavor differences existed among the 3 chosen varieties.

EXPERIMENTAL

Berries from the 3 varieties were available from the Horticulture gardens, growing side by side in single unreplicated rows, 100 feet long.

The study, for flavor only, comprised 4 replicates of complete blocks by a preference ranking method, 4 complete blocks by a method which involved checking flavor in relation to an "average" rating, and 8 repetitions of pairs in a completely balanced incomplete block design which included stating preferences as well as the degree of difference between the pairs.

Berries from half of each row were harvested daily, washed in cold running tap water, capped, and sliced as needed. Presentations within the complete block samples were randomized. Pairs were presented 6 at a time, with 3 combinations randomized and the other 3 reversed. This constituted 2 repetitions of each pair, and the presentation was repeated 4 times. Tasters at 2 locations judged the berries on alternate days. A replicate of each of the 3 methods was completed on one day. Thus replicate 1 of terms, ranks, and pairs was tested on one day's strawberry harvest and should be strictly comparable.

The berries were served in pink paper bakers (to mask color differences) to the volunteer judges in their respective offices.

Thirty-four experienced, but untrained, judges participated in the test. In the final analyses, occasional judges were dropped from one or another of the tests for reasons of missing blocks or for errors or omissions on the record forms.

In the ranking test, each judge was asked to rank the 3 samples in order of preference for flavor (Figure 1). In the "term" test, the judge checked the term which he believed

FLAVOR EVALUATION
RATE FLAVOR ONLY. IGNORE ALL OTHER DIFFERENCES

Name_____ Date_____

Instructions:

Rank the samples with the one you like best in 1st place and the one you like least in last place. No ties should be given.

	Write Sample Number Flavor	Check Are samples acceptable Yes 2	No. 1
1st (best)	_____ +.85	_____	_____
2nd	_____ 0	_____	_____
3rd (poorest)	_____ −.85	_____	_____

Figure 1.

described the flavor of each of the 3 samples—*average, above average* to a slight, moderate, or marked degree, and *below average* to the same extent (Figure 2). There was no reference sample and so each judge had to relate *average* to his past taste experience or flavor memory. The "term" test was included because it was being considered for use in a regional study, and there was a real need to test its efficacy against other techniques.

In the paired test, the judge was asked to check his preference for a pair member (essentially ranking) and was further requested to indicate the degree of difference—

FLAVOR EVALUATION
RATE FLAVOR ONLY. IGNORE ALL OTHER DIFFERENCES

Name_____ Date_____

Check the term which you think best describes the *flavor* of the sample.

	Above Average			Average	Below Average		
	Large	Moderate	Slight	In flavor	Slight	Moderate	Large
Sample A							
Sample B							
Sample C							
	+3	+2	+1	0	−1	−2	−3

Figure 2.

slight, moderate, marked or *none* (Figure 3). The scores assigned to each category are indicated in Figures 1 to 3. These scores were not known to the judges.

The ranked data were transformed to scores according to Fisher and Yates *(10)* for normalizing ranked data and were analyzed by the analysis of variance method *(2)*.

The "term" data were assigned scores from +3 for the highest rating above average to −3 for the lowest rating. These scores were also analyzed by the variance method.

The paired data were analyzed by 3 methods: (A) The preferences were handled as ranked data, submitting the transformed scores to an analysis of variance; (B) the preferences were also analyzed using the method of Terry, Bradley, and Davis *(17)*, for handling paired comparisons, which eschews variance techniques and measures sample differences by chi square; (C) the quantitative or intensity data resulting from the paired test were assigned scores on a 7-point scale, +3 to −3, and submitted to the analysis of variance method according to a technique devised by Scheffé *(16)*.

RESULTS AND DISCUSSION

The raw data, the arrays of mean scores with F tables, the differences, and the chi squares are shown in the tables.

Table 3 shows 3 arrays of means resulting from ranks and terms in complete blocks, and preferences by pairs in incomplete blocks. By all of these

FLAVOR EVALUATION
RATE FLAVOR ONLY. IGNORE ALL OTHER DIFFERENCES

Name_____ Date_____

Instructions:

Compare each pair separately. Taste in order: Pair 1, 2, 3. Always taste sample A before sample B.

	Check Preference		Check One The difference is:			
	A	B	Large	Moderate	Slight	None
Pair No. 1						
Pair No. 2						
Pair No. 3						
	+0.56	−0.56	+3 or −3	+2 or −2	+1 or −1	0

Figure 3.

TABLE 1
Raw data strawberries

Variety	Rep.	Terms Relating to "Average" Votes by 32 Judges. 4 Replicates. Complete Blocks							Ranking Preferences of 33 Judges 4 Replicates. Complete Blocks		
		Above Average:				Below Average:			Placement		
		Large	Moderate	Slight	Average	Slight	Moderate	Large	First	Second	Third
Sparkle	1	2	8	6	9	4	0	3	20	3	10
	2	0	5	9	13	3	2	0	15	7	11
	3	0	3	7	12	7	2	1	9	12	12
	4	0	7	4	17	3	1	0	18	9	6
Total		2	23	26	51	17	5	4	62	31	39
Monmouth	1	1	5	6	11	6	3	0	7	11	15
	2	1	3	1	16	6	5	0	16	12	5
	3	1	7	5	15	4	0	0	16	12	5
	4	0	2	6	8	12	3	1	9	16	8
Total		3	17	18	50	28	11	1	48	51	33
Orland	1	0	1	5	18	6	2	0	6	19	8
	2	3	3	5	10	8	3	0	2	14	17
	3	0	1	3	9	14	5	0	8	9	16
	4	0	2	3	7	12	6	2	6	8	19
Total		3	7	16	44	40	16	2	22	50	60

methods the flavor differences between Sparkle x Orland and between Monmouth x Orland were shown, but by *only* the paired method was the difference between Sparkle x Monmouth demonstrable.

At this point, we reached an impasse as to the precision evaluation of the 3 methods. It was deemed that the magnitude of the F values was not a valid criterion to judge efficiency of method especially when different scales of measurement were used *(7)*. An advantage in terms of number of replicates was attached to the paired data which comprised 8 replications as opposed to 4 only for each of the other two methods. To offset this advantage and to reduce the experiments to more comparable terms, the paired comparison data were segregated into two groups of 4 replicates each, on the basis of first and second appearance each day with the restriction that the order of presentation was balanced (Table 4).

A simple comparison of the number of significantly different varieties (or treatments), in the final array of means has been used as a basis to judge method precision *(4, 8, 12)*. If one method yields 4 significantly different treatments while a second yields only 2, the former is considered the more precise.

If this yardstick were to be used on the strawberry data, we would conclude that the paired method is the most precise because therein we were able to demonstrate 3 significantly different varietal flavors by variance analysis whereas, with the ranking and term techniques, only 2 flavor levels were demonstrable.

TABLE 2
Raw Data-Strawberries-Paired Comparison [1]
8 replicates: 4 random, 4 reversed [2]

		Frequency of Votes									
		Preference 34 Judges		Intensity Scale 33 Judges							
Variety	Rep.[3]	Sparkle	Monmouth	+3	+2	+1	0	−1	−2	−3	Total Score Favor of: Sparkle
Sparkle vs. Monmouth	1	19	15	2	11	5		9	5	1	+11
	2	17	17	3	5	8		9	4	4	− 2
	3	17	17	3	8	5	2	6	9		+ 6
	4	19	15	1	8	6	5	6	7		+ 5
	5	20	14	5	10	3	3	8	3	1	+21
	6	21	13	1	6	12	4	8	2		+15
	7	21	13	2	9	8	1	9	4		+15
	8	18	16	2	5	7	3	6	8	2	− 5
Total		152	120	19	62	54	18	61	42	8	+66
		Sparkle	Orland								Sparkle
Sparkle vs. Orland	1	22	12	8	10	2	2	8	2	1	+31
	2	24	10	7	6	10	1	2	6	1	+26
	3	27	7	8	9	8	1	6	1		+42
	4	27	7	4	9	13	1	3	2	1	+33
	5	24	10	5	9	9		6	4		+28
	6	29	5	4	14	10	1	2	2		+44
	7	30	4	8	10	10	1	1	3		+47
	8	29	5	7	9	11	1	3	2		+43
Total		212	60	51	76	73	8	31	22	3	+294
		Monmouth	Orland								Monmouth
Monmouth vs. Orland	1	20	14	4	9	5	2	7	6		+16
	2	22	12	3	8	9	2	5	5	1	+16
	3	24	10	3	11	8	3	3	4	1	+25
	4	25	9	6	9	9		4	4	1	+30
	5	21	13	1	4	14	3	7	2	2	+ 8
	6	24	10	3	9	10	3	3	5		+24
	7	21	13	2	12	6	1	8	3	1	+19
	8	22	12	4	6	8	4	5	4	2	+13
Total		179	93	26	68	69	18	42	33	8	+151

[1] Method from Scheffé, ref. 15.
[2] No significance was attached to order of presentation.
[3] Two replicates (1, 2; 3, 4; 5, 6; 7, 8) presented at one time.

To carry the pattern a step further, we should decide that the ranking and term methods were similarly precise because each showed only one significantly different variety. We could also decide that the simple preferences in the paired comparisons gave as much (set 1) or more (set 2) information as the intensity scale (Table 4) (degree of sample difference) and, on this basis, we could dispense with the scale in the interests of simplicity.

This approach leaves no avenue by which to judge the merits of 2 techniques which result in the same number of significantly different varieties (e.g. ranking and terms). Neither does the approach permit the evaluation of

TABLE 3

Flavor evaluation. Strawberries. July 1955

TERMS				RANKING			RANKING. PAIRS		
Complete Block. 4 Replicates				Complete Block. 4 Replicates			Balanced Incomplete Block 8 Replicates		
Variety			*Total Scores*[1]	*Variety*		*Total Scores*[2]	*Variety*		*Total Scores*[3]
Sparkle			+39	Sparkle		+19.55	Sparkle		+103.04
Monmouth			+ 8	Monmouth		+12.75	Monmouth		+ 30.24
Orland			−39	Orland		−32.30	Orland		−133.28
L.S.D.	.05		45		.05	28.34		.05	46.18
	.01		60		.01	37.69		.01	61.42
Source	*D.F.*	*M.Sq.*	*F*	*D.F.*	*M.Sq.*	*F*	*D.F.*	*M.Sq.*	*F*
Total	383			395			1631		
Variety	2	12.05	6.00**	2	6.02	7.92**	2	26.93	54.96**
Reps.	3	3.24	2.63	3			7		
Judge	31	2.15	1.75*	32			33		
Var. X Judge (error for Var.)	62	2.01	1.63**	64	0.76	1.85**	66	0.49	1.75**
Var. X Rep.	6	4.77	3.88**	6	1.78	4.34**	14	0.22	
Error	279	1.23		288	0.41		1509	0.28	

[1] Range + 3 (markedly above average) to− − 3 (markedly below average); 32 judges. 3 sig. (5% or better).
[2] Ranks transformed to scores (10) 33 judges; 6 sig. (5% or better).
[3] Ranks transformed to scores (10); 34 judges; 20 sig. (5% or better).

TABLE 4

Analyses of paired comparisons

Variety	Ranking[1] Sets of 4 Replicates 34 Judges Set 1 Total Score	Set 2 Total Score
Sparkle	+49.28	+53.76
Monmouth	+10.08	+20.16
Orland	−59.36	−73.92
L.S.D. .05	29.82	29.86
.01	39.66	39.71

Source	*D.F.*	*M.Sq.*	*F*	*M.Sq.*	*F*
Variety	2	11.13	27.15**	16.10	39.27**
Variety x Judge (error for Var.)	66	0.41	1.41*	0.41	1.46*
Variety x Rep.	6	0.45	1.55	0.23	.....
Error	705	0.29		0.28	

Variety	Intensity Scale[2] Eight replicates and two sets of 4 replicates 33 Judges 8 replicates	Set 1	Set 2
Sparkle $\hat{a}_1$	+0.4545	+0.5076	+0.4015
Monmouth $\hat{a}_2$	+0.1073	+0.0379	+0.1768
Orland $\hat{a}_3$	−0.5619	−0.5455	−0.5783
Y.05	0.1973	0.2810	0.2765
Y.01	0.2465	0.3513	0.3456

Source	*D.F.*	*M.Sq.*	*D.F.*	*M.Sq.*	*M.Sq.*
Main Effects	2	211.38	2	110.22	104.33
Deviations from Subtractivity	1	7.50	1	1.80	6.28
Order Effects	3	1.01	3	3.59	0.55
Error	786	2.73	390	2.77	2.68

[1] Preferences transformed to scores (10) and analyzed by variance.
[2] Degree of preference between pairs analyzed according to Scheffé *(15)*.

2 methods which show no significant differences. Such methods could conceivably differ in sensitivity and have prognostic value for future testing if relative precision could be resolved.

We next wondered if the degree of significance levels (5% vs. 1%) and/or the number of individual judges who detected significant differences could be used as a measure of method precision. By the term technique Monmouth was preferred to Orland at the 5% level. By ranking and paired comparison the same preference was significant at the 1% level.

It would seem that the number of discerning judges would indicate at least the ease with which differences may be detected.

Variance analyses or analyses from the range *(18)* of the 32–34 individual judge records showed that the number detecting significant varietal differences were:

Method	Number of "Significant" Judges 5% or better
Terms, 4 replicates	3
Ranks, 4 replicates	6
Pairs, 1st set of 4 replicates	13
Pairs, 2nd set of 4 replicates	15
Pairs, 1st and 2nd set combined, 8 replicates	20

If these observations have any value as criteria of precision, they would indicate that paired comparisons are most sensitive, ranking next, and terms least. By the same token, it would seem that 8 replicates would give more precise information than 4 in the paired comparison. In addition, with 8 replicates the Sparkle-Monmouth flavor difference was more highly significant ($P = .01$) than with each of the sets of four replicates ($P = .05$) when preferences only were considered.

When the records describing degree of difference between paired samples were analyzed by the method of Scheffé *(16)*, the resulting means ($\hat{a}$) from 8 replicates demonstrated differences ($P = .01$) between all pairs and thereby agreed with the results of the simple preferences (Table 4).

Agreement between preference and intensity was less consistent in the sets of 4 replicates. In set 1, the Sparkle-Monmouth difference was demonstrated from the preference records ($P = .05$) as well as from the intensity records ($P = .01$). In set 2, a similar difference was shown for preference ($P = .05$) but no significance was attached to the Sparkle-Monmouth difference in flavor intensity (Table 4). A re-analysis of the intensity records for Set 2 using transformed scores—a suggestion by Bliss *(1)* to offset the effect of jamming at the ends of the scale and of a scarcity of zeros—produced no change in the significance levels between varieties.

An examination of the raw data showed that in Set 1, the 33 judges preferred Sparkle to Monmouth 74 from 132 times with a total degree of preference at +54. In Set 2, Sparkle was preferred 71 from 132 times but the degree of preference was only +9. There is no explanation for the discrepancy as each replicate pair of Set 1 and Set 2 was presented at the same time from the same day's strawberry harvest.

The analyses indicated that the Sparkle-Monmouth difference was rather tenuous and that maximum discrimination, a forced choice, or increased

replication was required to detect the small qualitative distinction between the two. An intrinsic advantage was attached to even 4 replicates of the paired comparisons because each replication represented 2 tastings of each variety as compared to one tasting per replication for the complete block designs. In any comparison of techniques, the extra tastings should be considered.

In all of these analyses except pairs, Set 1 (intensity), the variety × judge interaction was significant (5% or better). As the interaction mean square was used for error to determine variety F values, our conclusions regarding differences seemed justifiable. These interaction significances seemed to us could be caused by: (a) large numbers of non-discriminating judges expressing randomness; (b) disagreement among panel members in degree but not in kind; and (c) disagreement among panel members in the rank order of the varieties. The third reason seemed the only one to cause concern and to require a minority report. As the Sparkle-Monmouth difference was not significant in 2 of the 3 experiments, the cases where Sparkle was placed in third place are reported:

Method	Divergent Opinion, Sparkle in 3rd Place
Terms:	5 (1 significant) of 32 judges
Ranks:	6 (1 significant) of 33 judges
Pairs: Set 1	2 (0 significant) of 34 judges
Pairs: Set 2	2 (0 significant) of 34 judges

To investigate the extent to which these dissenting judges influenced the variety × judge interaction, their records were omitted in separate analyses of variance (Table 5). In each case when the interactions were significant, the omission of these judges effected a lowering of the F values for the interactions to a non-significant level. Thus, the significant interactions in the all-judge analyses were caused mainly by panel members disagreeing in the rank order of the varieties. In Table 5, all of the scores were changed to assign each system a mean of 4 with intervals of 1 in order to facilitate a comparison of means resulting from different methods.

To determine method precision, several approaches were investigated: coefficients of variability including numbers of replications needed for a given degree of precision *(15)*, error variance ratios *(5, 6)* and regression coefficients. All of these techniques were discarded because of questionable validity due to differing scale lengths or heterogeneous error variances.

Recently, Kramer *(11)* devised a technique to compare sensitivity of judge panels for which he employed the sample number ratio (SNR × N) and defined as the number of tastings (judge × tastings) required for significance at a chosen probability level. The method is explained in detail in a recent paper by Wiley *et al. (19)*.

The values are computed from the formula:

$$\text{SNR} = \left(\frac{\text{LSD treatment}_{.01}}{\text{Range of treatment means}} \right)^2$$

The tastings required are calculated from:

$$\text{SNR} \times \text{N} \text{ where N} = \text{total number of tastings used.}$$

TABLE 5

Certain comparisons among three methods for taste tests

Method (4 reps) and No. of Judges	Scale Mean: 4 Intervals: 1	Variety Means[1]	F Var. x Judge	No. of tastings used	S.N.R. X N	No. of "Best" Judges[2]	Variety Means[1]	F Var. X Judge	S.N.R. X N	Ratio of Efficiency (Terms = 1.00)	
										All Judges	Best Judges
Term: 32	1 to 7	Sp. 4.30 Mon. 4.06 Orl. 3.70	1.63**	384	235	27	Sp. 4.47 Mon. 4.00 Orl. 3.56	1.36	87	1.00	1.00
Rank: 33	3 to 5	Sp. 4.17 Mon. 4.11 Orl. 3.71	1.85**	396	217	27	Sp. 4.32 Mon. 4.06 Orl. 3.62	1.34	72	1.08	1.21
Pairs: 34 Preferences Set 1	3.5 to 4.5	Sp. 4.16 Mon. 4.03 Orl. 3.81	1.41*	816	113	32	Sp. 4.18 Mon. 4.02 Orl. 3.79	1.25	86	2.08	1.01
34 Set 2	3.5 to 4.5	Sp. 4.18 Mon. 4.07 Orl. 3.76	1.46**	816	78	32	Sp. 4.84 Mon. 4.02 Orl. 3.13	1.27	53	3.01	1.64
Pairs: 33 Intensity Set 1	1 to 7	Sp. 4.69 Mon. 4.17 Orl. 3.14	1.21	792	115	30	Sp. 4.91 Mon. 4.18 Orl. 3.14	1.04	80	2.04	1.09
33 Set 2	1 to 7	Sp. 4.58 Mon. 4.20 Orl. 3.14	1.15	792	133	32	Sp. 4.62 Mon. 4.22 Orl. 3.09	1.18	123	1.77	0.71

[1] Dividing lines indicate significant differences: single = 5%; double = 1%.
[2] Best judges: eliminating those who rated Sparkle in third place.

The N, therefore, includes the double number of tastings by the paired comparison method as opposed to the complete block choices.

After a fruitless search for statistical techniques to compare efficiency among methods involving different scales, this SNR technique appeared to be applicable and was considered so by the author *(11)*. In consequence, the method was applied to our data (Table 5). The $LSD_{.01}$ were based on the interactions, variety × judge, because they were used instead of error to calculate variety F. In addition, the SNR were calculated from the "best judge" analyses (eliminating those who chose Sparkle in third place) to demonstrate the effect of non-consistent judges upon these values (Table 5).

If these evaluations are valid and appropriate to compare method precision, they indicate that the paired comparisons were approximately 2 to 3 times as efficient as the term and rank methods.

When the records of dissenting judges were omitted, however, the SNR indicated only slight differences in method efficiency. In addition, the Sparkle-Monmouth differences by the term and ranking methods were increased to a significant level by eliminating the 5- and 6-judge records. In set 2 (intensity) dropping one judge was not sufficient to demonstrate the Sparkle-Monmouth difference.

The acceptability records from the ranking (complete block) test were assigned scores of two for *acceptable* and one for *not acceptable* and submitted to an analysis of variance. There was no significance attached to the results. The average scores also indicated a high degree of acceptability (Table 5) for all varieties.

Of interest was the preference of the judges for a specified method. Records from 31 judges showed that 24 preferred to judge by paired comparisons, 4 by ranking, 2 by terms and 1 no preference.

The paired data were also analyzed according to Terry *et al. (17)*. This demonstrated significant differences among varieties (Table 6) and significant disagreements among panel members. By this method, it was not possible to

TABLE 6
Analyses of pairs

All 33 Judges. 8 Repetitions
Analyzed According to Terry et al. (16)

	Sparkle Scores = 880	*Monmouth* 820	*Oriand* 676
Test 1.	Assumed judges were consistent to panel. d.f. = t-1 = 2 chi square = 11.2** Large chi square value indicated true treatment differences.		
Test 2.	Assumed judges were consistent only as individuals. d.f. = g (t-1) = 66 chi square = 202.3** Large chi square value indicated true treatment differences.		
Test 3.	Test of agreement among panel members. d.f. = 32(2) = 64 chi square = 90.7** Large chi square value indicated panel disagreement.		

know whether or not Sparkle differed significantly from Monmouth. No attempt was made to relate the findings to the results from the other analytical methods.

Another dire need which confronts the food quality investigator is a method to evaluate the precision of different sized panels judging the same foods. The moot question appears to be: Is a small panel as good or better than a large panel? A small panel which can give accurate answers is a consummation devoutly to be wished. It is generally believed that the verdict of small panels of 4–6 discriminating judges is a better indicator of differences than that of a large (30–40) randomly-selected group. Experimental evidence on this score is lacking and simple valid statistical techniques are needed to compare the performances of different panels.

In this laboratory, a running check of the significance of individual judges over a period of years *(13)* indicates that it is impossible to predict the performance of even the usually acute judge, since, upon occasion, he may be totally unreliable. If he represents 1/6 of the panel, his performance affects disproportionately the consensus regarding sample differences.

Variance analyses or analyses from the range of individual judge records were used as criteria of taste acuity: if a judge detected differences, in any direction, at the 5% level or better, he was considered an acute judge.

For purposes of illustration only, judges were randomly selected from our 34 people (paired comparisons, 8 replications) to simulate smaller panels. Five panels, here considered *bona fide* panels and not *a posteriori* selections, were randomly chosen within the significant and non-significant groups of judges with one exception which was randomly selected from all judges. The intensity data were analyzed by the Scheffé technique *(16)*. The preference data, distributed around a mean of 4, were treated by variance analyses (Table 7).

It appears that in no case did the smaller panels detect the Sparkle-Monmouth difference to the same degree ($P = .01$) that was perceived by the all-judge panel with 8 replications (Table 3, preference; Table 4, intensity).

The SNR × N varied from 34 to 576 indicating large differences in the efficiency of the several panels. Two discrepancies as to significantly different varieties occurred between the two scales. With 8 significant judges, the intensity scale showed a Sparkle-Monmouth difference ($P = .05$) which was not apparent from the preference data. The 3-judge panel detected a Monmouth-Orland difference ($P = .01$) by means of the intensity scale but the same panel detected no difference whatsoever between varieties by the preference ratings. If detection of differences and levels of significance are criteria of precision, in these two instances the intensity scale would be more precise than the ranking in pairs.

The panel of 8 significant judges with 384 tastings detected no difference between Sparkle and Monmouth, but the tastings required (SNR × N) were only 34. The all-judge panel of 34 with 1632 tastings detected the Sparkle-Monmouth difference ($P = .01$) while the tastings required were 107.

This discrepancy between tastings required and significance levels was investigated further by determining the distribution of SNR × N among significant-judge panels of 8. The 21 significant judges were listed by random

TABLE 7

Comparison of panels of different sizes and compositions[1]

From paired comparisons, 8 replications

Variety	8 Significant Judges		8 Judges 5 Significant		8 Judges 3 Significant[2]		5 Judges 3 Significant		3 Judges 2 Significant	
	Choice Mean[3] Scores	*Intensity* $\bar{A}$	*Choice* Mean Scores	*Intensity* $\bar{A}$	*Choice* Mean Scores	*Intensity* $\bar{A}$	*Choice* Mean Scores	*Intensity* $\bar{A}$	*Choice* Mean Scores	*Intensity* $\bar{A}$
Sparkle	4.22	+.6146	4.16	+.4167	4.12	+.4271	4.12	+.2917	4.12	+.3194
Monmouth	4.11	+.2083	4.05	+.1041	4.08	+.1510	4.11	+.3500	4.12	+.2639
Orland	3.67	−.8229	3.78	−.5208	3.80	−.5781	3.76	−.6417	3.75	−.5833
L.S.D. or Y .05	0.12	.3857	0.19	.3904	0.19	.4072	0.23	.5389	N.S.	.5817
.01	0.17	.4822	0.26	.4880	0.26	.5090	0.34	.6800	N.S.	.7340
S.N.R. x N	34		180		254		214		576	
Source		*M.Sq.*		*M.Sq.*		*M.Sq*		*M.Sq.*		*M.Sq.*
Variety or Main effects	5.36	105.44	2.49	43.74	2.02	51.78	1.70	37.16	1.12	18.43
Variety x Judge (error for Var.)	0.10		0.25		0.24		0.20		0.31	
Deviations from Subtractivity		0.44		1.69		1.51		0.19		0.35
Order effects		0.78		0.36		1.40		1.58		0.90
Error	0.10	2.53	0.11	2.59	0.12	2.82	0.10	3.02	0.12	2.11

[1] Randomly selected within significant and non-significant judges.
[2] Randomly selected from all judges.
[3] Choices assigned values of 3.5 and 4.5 (Mean 4.0).

selection and segregated into 14 panels of 8 each chosen as follows: Panel 1 included judges 1 to 8; Panel 2, 2, to 9; Panel 3, 3 to 10, etc. All of the data from these panels were submitted to analyses of variance. The tastings required ranged from 16 to 86 (Table 8) and averaged 53. Only Panel 1 demonstrated a significant variety × judge interaction. In all panels except number 3, the varieties Sparkle and Monmouth were judged to be different to a significant degree.

If the SNR × N is a realistic criterion of panel precision, the paired comparison method with panels of 8 acute judges appeared to be approximately twice as precise (required tastings: 53) as the randomly selected panel of 34 using the same method (required tastings: 107). None of the small panels which included non-discriminating judges appeared to be as precise (180 to 576) as the all-judge group.

SUMMARY

Three strawberry varieties believed to represent three flavor levels were tested organoleptically by ranking in complete blocks, four replicates; checking against a preconceived average (called "terms") in complete blocks, four replicates; and by paired comparisons, with records on degree of difference, in completely balanced incomplete blocks, eight replicates. The varieties and characteristic flavor levels were: Sparkle, high; Monmouth, medium; Orland, low.

The resulting data were analyzed by variance analyses using transformed scores for the ranked data (pairs and complete blocks), assigned scores for the term data, a modified variance analysis for the intensity scale, and a specified chi square analysis for the paired preferences.

If the number of significantly different varieties, the significance level of the differences, and the sensitivity of the judges to flavor differences may be used as criteria to appraise adequacy or superiority of a technique, the paired comparisons with eight replications would be considered the most sensitive because only by this method was there demonstrated a quality difference between Sparke and Monmouth ($P = .01$). Moreover, by these criteria, the simple preference gave as much information as the intensity scale.

When the eight replications were reduced to two balanced sets of four replicates, analyses of the preferences (paired) still indicated a Sparkle-Monmouth difference in both sets but at a lower significance level ($P = .05$). In the second balanced set of four, discrepant results between the intensity and preference scales appeared and no Sparkle-Monmouth difference was apparent from the intensity ratings.

An attempt to rate precision by calculating the number of tastings required based on the sample number ratio resulted in approximate efficiency ratings of ranks equally as efficient and paired comparisons two to three times as efficient as terms.

Whether or not the foregoing criteria are valid and realistic in reflecting the true relative precision of the methods is not known. The evidence, however, appears to favor paired comparisons over ranking and terms.[b]

[b] It was not the purpose of this study to attempt to explain why one method was superior to another. Our objective was solely an attempt to compare three specific methods which are described in detail.

TABLE 8

Distribution of analytical data from 14 panels of 8 significant judges

Paired comparisons, 8 replications, choices only

Mean scores

Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sparkle	4.24	4.21	4.18	4.21	4.21	4.23	4.23	4.23	4.24	4.23	4.26	4.23	4.23	4.25
Monmouth	4.05	4.06	4.10	4.07	4.06	4.04	4.05	4.05	4.05	4.07	4.04	4.05	4.08	4.07
Orland	3.71	3.73	3.72	3.72	3.73	3.73	3.71	3.71	3.70	3.70	3.70	3.71	3.69	3.68
L.S.D. .05	0.18	0.15	0.13	0.13	0.15	0.12	0.13	0.13	0.13	0.12	0.12	0.11	0.08	0.08
.01	0.25	0.21	0.18	0.19	0.20	0.16	0.18	0.18	0.18	0.17	0.16	0.15	0.11	0.12
S.N.R. x N	86	74	59	58	67	40	46	46	43	40	32	32	16	17
Source D.F.	Mean Squares from Analyses of Variance Tables													
Variety[1] 2	4.62	3.94	3.90	4.12	3.94	3.95	4.52	4.52	4.80	4.88	5.00	4.52	5.08	5.44
Variety x Judge[2] 14	.22**	.16	.12	.13	.15	.09	.11	.11	.11	.10	.09	.09	.05	.05
(error for var.)														
Error 161	.10	.11	.11	.11	.11	.11	.10	.10	.11	.11	.11	.11	.11	.11

[1] All panels showed significant variety differences (P = .01).
[2] Only panel 1 showed significant interaction (P = .01).
[3] Dividing lines show significant differences: Single = P = .05; Double = P = .01.

In all cases, the calculated required tastings indicated that fewer tastings were needed than were actually used. This indication was not realistic since the Sparkle-Monmouth difference was not always demonstrated.

Variance analyses of data on acceptability lacked significance, which implied that although Sparkle and Monmouth were significantly preferred to Orland, fresh berries of the Orland variety were quite acceptable to the panel of 33 judges.

Analyses of the paired data recorded by groups of three to eight judges who were randomly selected from the 33 people to simulate five small panels showed that none of the small panels consistently perceived the Sparkle-Monmouth difference to a significant degree. If precision is judged solely on the basis of SNR $\times$ N, however, the panel of eight significant judges would be considered the most efficient (better than 34 judges, 8 replications) although the Sparkle-Monmouth difference was not detected by this panel.

Analyses of the paired data from 14 panels of eight significant judges showed that all but one of these detected the Sparkle-Monmouth difference, with fewer tastings required than for the 34-judge group. If conclusions are drawn from the number of statistically different varieties, 13 of the significant-judge small panels and the large panel were equally sensitive, while the large panel was a better indicator of differences than two of the acute-judge panels and all of the randomly-selected small panels. By the SNR criterion, all 15 of the acute eight-judge panels were more precise than the large panel. Ultimately, the problem would be the *a priori* selection of few judges with the ability to reflect the consensus of the larger group.

CONCLUSION

The intent of the investigator will govern decisions as to method, number of replications, and size of panel. It is conceded that factors other than precision may be important considerations.

The ranking and term techniques required an equal expenditure of material and time.

The time needed to judge three samples by the paired method was twice that required by the other two techniques and would be threefold for four samples and fourfold for five samples but the fewer number of tastings required might compensate completely for the time expenditure.

The investigator, therefore, must consider the need for the detection of small differences, the availability of uniform sample material (than which there is no factor more limiting) and the expense.

With regard to the strawberry varieties, our first conclusion was that all varieties were acceptable.

If our interest lies in wide quality differences and our motive is the desire to present to the consumer a high-quality berry, then we could recommend either Sparkle or Monmouth as being superior to Orland, and we could ignore the Sparkle-Monmouth difference as not apparent to the usual consumer of fresh berries. These conclusions may be gathered either from the test by terms or by ranks.

If our motive is to improve the intrinsic flavor of berries and to present to the consumer the highest quality possible to achieve, then we are interested

in the Sparkle-Monmouth difference, detectable only by paired comparisons. In this event, we should recommend Sparkle or we should be over-ruled by other characteristics, e.g. winter hardiness or adaptability to a specific environment.

Small differences are of interest to the geneticist whose objectives include superior quality. The entomologist or pathologist is also interested in small flavor differences associated with pesticides in order to improve his formulations and to determine the frequency of applications.

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IDENTIFICATION OF THE VOLATILE SULFUR COMPONENTS OF COOKED CABBAGE AND THE NATURE OF THE PRECURSORS IN THE FRESH VEGETABLE [a]

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It is well known that cabbage contains thermolabile sulfur compounds which evolve sulfurous odors. Simpson and Halliday *(22)* investigated the volatile sulfur produced by boiling cabbage during the period of cooking. The formation of hydrogen sulfide was attributed to the hydrolysis of allyl isothiocyanate, the mustard oil of the thioglucoside sinigrin. Masters and Garbutt *(16)* similarly described the volatile sulfur as sulfide sulfur, but made no identification of the volatile organic sulfur.

The present investigation identifies the volatile divalent sulfur compounds of cooked cabbage. For this purpose a train of absorption traps is utilized modifying the procedure of Challenger and Rawlings *(7)*.

It is also shown that the major volatile sulfur component of cooked cabbage is derived from L-S-methylcysteine sulfoxide, a free amino acid occurring in cabbage, first described by Synge and Wood *(24)*, and reported to account for a substantial proportion of the organic sulfur of cabbage.

The relationship between thermolabile precursors and volatile sulfur is of considerable importance to flavor, if one accepts the supposition that odors commonly described as disagreeable may contribute to an acceptable flavor, and be considered normal constituents of the aroma, when present in trace amounts. Examples of such a possible role for sulfur compounds are: hydrogen sulfide and mercaptans in beer *(5)*, the brothlike flavor of 3-methylmercaptopropionaldehyde *(17)*, ethyl 3-methylthiopropionate in pineapple *(12)*, allyl isothiocyanate in mustard *(11)*, etc.

EXPERIMENTAL

Volatile sulfur, train analysis. The train of traps illustrated in Figure 1 was used for qualitative identification of volatile sulfur compounds. A head of cabbage (600 g.) was dispersed in a Waring blender with 150 ml. of distilled water. A 250 g. aliquot of the slurry was placed in a 500 ml. round-bottom flask, to which was fitted an adapter with gas inlet and a 20 cm. Allihn condenser. The train of traps consisted of (a) anhydrous calcium chloride, (b) lead acetate (s.) ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$), (c) 4% mercuric cyanide (aq.), and (d) 3% mercuric chloride (aq.). The calcium chloride and lead acetate traps separated hydrogen sulfide from organic sulfur *(20)*. The slurry was aerated with commercially purified nitrogen at the rate of 100 ml./min. by applying a vacuum at the end of the train. After 6 hours no precipitate was observed in the mercury salt solutions. The slurry was then boiled under reflux and aeration continued overnight. Lead sulfide was formed in the lead acetate trap in a few minutes. In 20 to 50 minutes

[a] Presented at the 129th meeting of the American Chemical Society, Dallas, Texas, 11 April 1956.

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after boiling, a white precipitate appeared in the mercuric chloride trap. A moist lead acetate paper inserted before the mercuric cyanide absorption tube remained colorless. No precipitate was obtained in the mercuric cyanide trap, indicating the absence of volatile mercaptans. At the termination of the run, the calcium chloride and lead acetate traps were swept with nitrogen for a half hour, and the contents then extracted with sulfur-free benzene. No mercaptan, mercaptide, or sulfides were detectable, confirming Reith's *(20)* observations. The same results were obtained with purified air as with nitrogen.

The white precipitate in mercuric chloride was identified as the decomposition product of dimethyl disulfide. The material did not melt at 250° C., and a sublimate of mercuric chloride was observed. By treatment with 6 N hydrochloric acid, the odor of methyl mercaptan was evolved. The mercuric chloride precipitate was decomposed with dilute sodium hydroxide, forming yellow mercuric oxide, but no odor was detected, indicating the absence of volatile thioethers. Acidification of the alkaline solution gave the odor of methyl mercaptan. A portion of the mercuric chloride precipitate was heated with 3 N hydrochloric acid, and the liberated mercaptan passed by a nitrogen stream into mercuric cyanide (Figure 1).[4] The white mercuric cyanide precipitate was identified as the

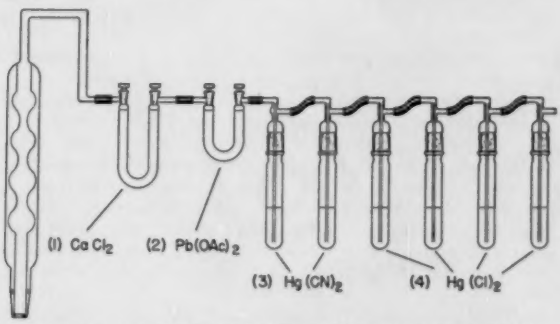

Figure 1. Absorption train for volatile sulfur compounds.

mercury mercaptide of methyl mercaptan. Recrystallization from alcohol yielded the pure mercaptide (m.p. 175° C., uncorr.). It did not lower the melting point of an authentic sample of mercury methylmercaptide (*Anal.* Calc'd for $C_2H_6S_2Hg$: C, 8.15; H, 2.05; S, 21.8. Found: C, 8.59; H, 2.11; S, 21.4). The mercuric chloride precipitate was heated with dilute sodium hydroxide, and aerated with nitrogen directly into 3% mercuric chloride. No precipitate was obtained, indicating the absence of organic sulfides.

The same procedure applied to the vegetables described in Table *2* yielded similar results. But a sample of sauerkraut gave a trace of methyl mercaptan during the early stages of heating.*

Distribution of sulfur in cabbage. A sample of cabbage (Table 1) was analyzed for total, volatile, and non-volatile sulfur. Two kilograms of domestic white cabbage was boiled under reflux, and aerated with nitrogen for 120 hrs. Five traps containing 3% mercuric chloride were used in the train of Figure 1. At the end of the run, no precipitate was formed in a fresh mercuric chloride trap introduced after the mercuric cyanide absorption tube during a period of 6 hours. The weight of precipitate (50 mg.) in the fifth mercuric chloride trap was small, compared to the total weight of precipitate obtained (3.15 g.). The mercuric chloride precipitate was collected, washed with water, and dried with alcohol and ether. An aliquot was heated with 3 N hydrochloric acid

[4] A trace of lead sulfide was also obtained from the mercuric chloride precipitate, indicating a trace of dimethyl trisulfide *(3)*.

* Ascorbic acid is added to delay darkening of non-heat sterilized sauerkraut, packaged in plastic bags *(2)*.

under reflux, and aerated with nitrogen. The methyl mercaptan evolved was absorbed in mercuric cyanide solution, traces of hydrogen sulfide being removed as in the train of Figure 1. The amount of sulfur occurring as dimethyl disulfide was calculated from the weight of mercury mercaptide obtained and the stoichiometry of the following reactions:

(1) $2CH_3SSCH_3 + (3x + 3)\ HgCl_2 + 2H_2O \rightarrow 3CH_3SHgCl.\ x\ HgCl_2 + CH_3SO_2H + 3HCl$

(2) $CH_3SHgCl \cdot xHgCl_2 + HCl \rightarrow CH_3SH + (x + 1)\ HgCl_2$

(3) $2CH_3SH + Hg(CN)_2 \rightarrow (CH_3S)_2Hg + 2HCN$

The amount of hydrogen sulfide sulfur was most conveniently obtained gravimetrically, by dissolving the lead acetate and conversion of the insoluble sulfide to lead sulfate. Non-volatile sulfur was determined in duplicate on aliquots of the total cooked cabbage slurry, evaporated to dryness.

Quantitative estimation of dimethyl disulfide. Several varieties of cabbage (Table 2) were investigated, and quantitative estimation of the disulfide sulfur obtained, by modifying the Segal and Starkey *(21)* procedure. The train consisted of the sequence: (a) calcium chloride, (b) lead acetate (s.), mercuric cyanide, (c) benzene with condenser, and (d) mercuric chloride. The amount of disulfide absorbed in benzene was estimated quantitatively by reduction with zinc and acetic acid, and Volhard titration of the liberated mercaptan. The recovery of authentic dimethyl disulfide from an aqueous suspension was quantitative, with results within 5% of the theoretical. Aqueous slurries of the vegetable materials were prepared by dispersion in a Waring blender. Moisture was determined in duplicate by lyophilization and vacuum desiccation *(15)*. A 500 g. aliquot of slurry corresponding to 35 g. (dry weight) of vegetable was boiled under reflux and aerated 40–60 hours at the rate of 100 ml./min. The termination of the evolution of dimethyl disulfide was checked by introducing a mercuric chloride trap in place of the benzene absorption bottle, and continuing aeration for six hours. Absorption of dimethyl disulfide in benzene was shown to be quantitative by absence of precipitate in the mercuric chloride trap at the end of the train. Additional confirmation of the identification of dimethyl disulfide was the formation of the yellow silver mercaptide duirng the reduction of the benzene solution.

Hydrogen sulfide was determined as before, and total sulfur obtained on aliquots of the dried vegetable by tetrahydroxyquinone titration of sulfate produced by microcarius combustion.

Fractionation to isolate precursor of dimethyl disulfide. Fresh cabbage was shredded, frozen with dry ice, and macerated. The minced material was extracted with aqueous alcohol (60% alcohol, based on moisture content of 90%). The extract was concentrated under vacuum at 33° C. The concentrate was defatted by extraction with ether, and evaporated to a syrupy residue, in a rotary-type vacuum still. The alcohol-insoluble material was obtained by repeated precipitation from aqueous solution. Most of the precursor of dimethyl disulfide was shown by train analysis (Figure 1) to be present in this fraction. A 3 g. aliquot of the alcohol-insoluble fraction, concentrated by vacuum distillation, was diluted to a volume of 25 ml., and the pH adjusted to 3.0 with 6 N hydrochloric acid. Displacement chromatography yielded "basic" and "neutral and acidic" fractions (Amberlite 105-H, 30–60 mesh; column 18 mm. x 17.5 cm.; elution with 0.2 N ammonium hydroxide).

L-S-Methylcysteine sulfoxide was detected in fractions "B" and "C" by paper chromatography (butanol-acetic acid-water, 4:1:5). S-Methylcysteine was likewise detected in the acid hydrolysate of fraction "C". The occurrence of the sulfoxide prior to acid hydrolysis is thus confirmed *(24)*.

Qualitative test for precursor of dimethyl disulfide. Fractions "A", "B", "C", and "D" (Figure 2) were shown to contain the precursor of dimethyl disulfide by train analysis. The same fractions formed methyl mercaptan when treated with 50% acetic acid and zinc dust. A 0.1 g. sample of vacuum concentrated material was dissolved in 1 ml. of water and 0.3–0.5 g. of zinc dust was added. The reaction mixture was heated to 100° C. in a test tube constricted at the top, and fitted by a rubber sleeve, with a U-tube containing 2 drops of 0.05 N silver nitrate. In 5–10 minutes, a yellow precipitate appeared in the silver nitrate solution. The precipitate was shown to be silver methylmercaptide by identifying the product of the zinc-acetic acid reaction as methyl mercaptan,

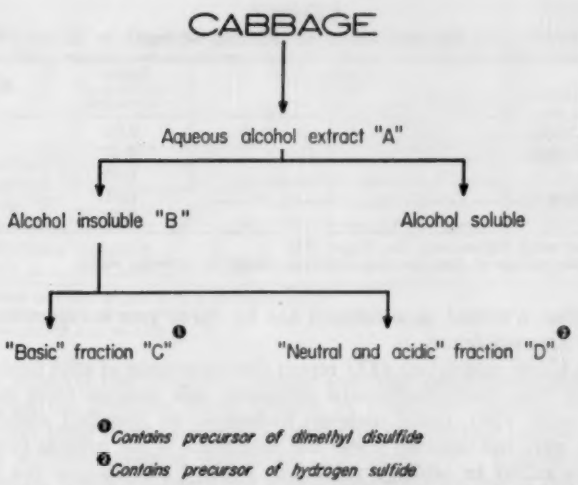
CABBAGE
Aqueous alcohol extract "A"
Alcohol insoluble "B"
Alcohol soluble
"Basic" fraction "C" ❶
"Neutral and acidic" fraction "D" ❷
❶ Contains precursor of dimethyl disulfide
❷ Contains precursor of hydrogen sulfide

Figure 2. Fractionation of cabbage to isolate precursor of dimethyl disulfide.

which, when absorbed in mercuric cyanide, yielded the mercury mercaptide. The sensitivity was 100 γ of sulfur at dilution of 1 : 1000 for dimethyl disulfide.

Formation of dimethyl disulfide from L-S-methylcysteine sulfoxide. A diastereoisomeric mixture of L-S-methylcysteine sulfoxide was synthesized according to the procedure of duVigneaud, Loring, and Croft *(8)* as adapted by Stoll and Seebeck *(23)*. The intermediate, L-S-methylcysteine [(m.p. 241–245° C. decomp., uncorr.) (*Anal.* Calc'd for $C_4H_9NO_2S$: N, 10.36. Found: N, 10.55)], yielded on oxidation with hydrogen peroxide L-S-methylcysteine sulfoxide (m.p. 173.5–173.8° C., uncorr.) from water-ethanol (*Anal.* Calc'd for $C_4H_9NO_3S$: N, 9.27. Found: N, 9.33).

The synthetic sulfoxide was refluxed 24 hours with 6 N hydrochloric acid. Paper chromatography (butanol-acetic acid-water, 4 : 1 : 5) showed complete destruction of the sulfoxide and the formation of S-methylcysteine *(24)*. The volatile sulfur was identified as dimethyl disulfide, by the train of Figure 1. No mercaptan or hydrogen sulfide was detected.

A 300 mg. sample of the synthetic sulfoxide was dissolved in 40 ml. of water. The solution was boiled under reflux. Aeration for 24 hours during reflux yielded a precipitate in mercuric chloride (Figure 1). No mercaptan or hydrogen sulfide was detected, and the mercuric chloride precipitate was identified as the decomposition product of dimethyl disulfide. Paper chromatography (butanol-acetic acid-water, 4 : 1 : 5) of the solution showed the presence of undecomposed sulfoxide, but no S-methylcysteine was detected. Pyruvic acid was identified as present by conversion to the 2,4-dinitrophenylhydrazone derivative, isolation by extraction, and comparison with a similarly formed sample of the 2,4-dinitrophenylhydrazone of authentic pyruvic acid, using infrared absorption and paper chromatography.

Qualitative test for L-S-methylcysteine sulfoxide. L-S-Methylcysteine sulfoxide reacted with zinc-acetic acid to form methyl mercaptan under conditions described for precursor-containing fractions. Cysteine, cystine, and methionine gave negative results. Methionine sulfoxide was reduced to methionine (detected by P. C.).

RESULTS AND DISCUSSION

Volatile sulfur of cooked cabbage. The major volatile sulfur constituents of cooked cabbage have been identified as dimethyl disulfide and hydrogen sulfide. Exhaustive cooking of a cabbage sample (Table 1) shows that the

TABLE 1
The distribution of sulfur in cabbage[1]

	Sulfur[2] g./100 g. of dry weight	Reference
Hydrogen sulfide	0.04	
Dimethyl disulfide	0.47	
Non-volatile S	0.30	*(1)*
Total (calculated)	0.81	
Total S	0.80	*(1)*

[1] Purchased retail, Philadelphia, Pa., March 1953.
[2] Results are average of duplicate determinations, except for hydrogen sulfide.

volatile sulfur obtained is accounted for by these two compounds. Methyl mercaptan does not occur.

Jensen, Conti, and Kjaer *(13)* report the occurrence of allyl isothiocyanate in cabbage. The methylmethionine sulfonium salt isolated from cabbage by McRorie *et al. (14)*, could undergo hydrolysis to dimethyl sulfide. These substances were not detected under the conditions of the present procedure.

Volatile sulfur in cabbage varieties. Table 2 summarizes the results on determination of dimethyl disulfide formed on exhaustively cooking varieties of *Brassica olearacea.*

Results for two different samples of cabbage (Tables 1 and 2) show that a variation in precursor sulfur exists. Peterson *(18)* attributes variation in sulfur of cabbage to environmental and seasonal conditions. Since the precursor of dimethyl disulfide is an amino acid, it is interesting to note that Peterson, Fred, and Viljoen *(19)* report a variation of 0.06–0.16% (fresh wt. of cabbage) in soluble nitrogen.

The precursor of dimethyl disulfide. L-S-Methylcysteine sulfoxide is demonstrated to be the precursor of dimethyl disulfide by the evidence summarized in Table 3.

A chemical study of precursor fractions of Figure 2 shows that acid hydrolysis yields S-methylcysteine and dimethyl disulfide. The precursor is water soluble, and is not a sulfhydryl or thiocarbonyl compound. Decomposition of precursor fractions to form dimethyl disulfide under neutral conditions does not yield S-methylcysteine. Under conditions of the zinc-acetic acid test methyl mercaptan is formed. The occurrence of S-methylcysteine sulfoxide in fraction "C" is sufficient to explain the above observations.

TABLE 2
Volatile sulfur in cabbage varieties[1]

	Moisture[2] %	Sulfur g./100 g. of dry wt.		
		Total S[2]	$(CH_3)_2S_2$[2]	H_2S
Cabbage	91.9	1.0	0.054[3]	0.003
Dehydrated cabbage	.....	1.2	0.030	0.003
Red cabbage	93.5	0.7	0.064	0.008
Sauerkraut	91.4	0.7	0.079	0.010
Cauliflower	89.6	1.3	0.183	0.002
Broccoli	88.2	1.3	0.150	0.001

[1] The fresh vegetables were purchased retail, Natick, Mass., summer of 1955.
[2] Average of duplicate determinations.
[3] Analysis by mercuric chloride precipitate (Figure 1) yielded 0.05 g./100 g. of dry wt.

TABLE 3

Formation of dimethyl disulfide from L-S-methylcysteine sulfoxide

	Train detection of $(CH_3)_2S_2$		Zn-HOAc test	NaNe-I_2[1] test	Ninhydrin test
	H_2O, 100° C.	6 N HCl 110° C.			
Cabbage extract "A"	+	+	+	+	−
Alcohol insoluble "B"	+	+	+	+	+
Basic fraction "C"	+[2]	+[2]	+	0	+
"Neutral and acidic" fraction "D"	0[2]	trace[2]	trace	+	0
L-S-methylcysteine sulfoxide (synthetic)	+[2]	+[2]	+	0	+

[1] Occurrence of −SH or >C = S is indicated *(9)*.
[2] No hydrogen sulfide was evolved.
[3] Hydrogen sulfide was evolved.

It was observel that S-methylcysteine sulfoxide decomposes more readily to form dimethyl disulfide when present in precursor fraction "C". It is proposed that some form of catalysis similar to the enzyme catalyzed decomposition of L-S-alkylcysteine sulfoxides is involved *(4)*. Elucidation of the mechanisms by which the precursor forms dimethyl disulfide is not necessary to the present demonstration that the sulfoxide is the main precursor of dimethyl disulfide. The trace of dimethyl disulfide occurring in fraction "D" may be formed from an intermediate in the reaction.

The precursor of hydrogen sulfide. Simpson and Halliday's *(22)* hypothesis that the hydrogen sulfide of cooked cabbage is formed from a thioglucoside is not inconsistent with the observations summarized in Table 3.

The odor of cooked cabbage. The odor of cooked cabbage may be attributed to the decomposition of L-S-methylcysteine sulfoxide to form dimethyl disulfide. Experiments to determine the total amount of disulfide formed from cooking cabbage show that such decomposition is a slow process. Hence, under normal cooking conditions, it is proposed that dimethyl disulfide is present in trace amounts, and contributes to normal aroma. Toennies and Kolbe *(25)* have described the odor of methionine and methionine sulfoxide as the "odor of boiled cabbage". The slow decomposition of such compounds in the solid state may form dimethyl disulfide. The role of L-S methylcysteine sulfoxide in the odor of cooked cabbage can be compared to that of S-allylcysteine sulfoxide and homologues in onion and garlic *(6, 23, 10)*.

SUMMARY

The volatile sulfur components of cooked cabbage have been identified as dimethyl disulfide and hydrogen sulfide. By analysis, dimethyl disulfide is the main constituent, accounting for the odor of cooked cabbage. Analytical data showing the occurrence of dimethyl disulfide in other varieties of cabbage, *Brassica oleracea,* is also reported.

The main precursor of dimethyl disulfide has been shown to be L-S-methylcysteine sulfoxide.

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ASCORBIC ACID OXIDASE ACTIVITY OF CERTAIN VEGETABLES AND CHANGES IN THE CONTENT OF REDUCED AND DEHYDROASCORBIC ACID DURING SHELF-LIFE [a]

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The majority of studies on ascorbic acid content of vegetables have been concerned only with the reduced form. Evidence is accumulating, however, which indicates that the dehydroascorbic acid contributes measurably to the total ascorbic acid content of certain vegetables *(1, 2, 3, 8, 10, 21, 22)*. The biological value of dehydroascorbic acid has been established as practically equal to that of reduced ascorbic acid and should be included in determinations evaluating plant materials as sources of Vitamin C.

This study involves the determination of the changes in the content of the reduced and dehydroascorbic acid of certain vegetables during a simulated shelf-life and the measurement of the activity of ascorbic acid oxidase, the enzyme system which catalyzes the oxidation from reduced to dehydroascorbic acid.

Limited data have been reported on changes in these two forms of ascorbic acid in vegetables during shelf life or storage. Zepplin and Elvehjem *(22)* reported the reduced and dehydroascorbic acid content of four vegetables over a 6-day holding period, the first 3 days of which the vegetables were held in crushed ice. Dodds *et al. (2)* reported on the reduced and dehydroascorbic acid content of the sweet potato at various intervals during storage. Several studies have been conducted which show that maceration of plant tissue increases the content of dehydroascorbic acid. These experiments involved short holding periods not exceeding 24 hours duration *(8, 16)*.

Enzyme systems capable of oxidizing reduced ascorbic acid directly have been found in a number of vegetables *(1, 6, 7, 17, 18, 19)*. Johnson and Zilva *(7)* have shown that in the potato, ascorbic acid is not oxidized directly but that the tissue contains phenolases which oxidize ascorbic acid in the presence of catechol or expressed juice. Huelin and Stephens *(5, 6)* have investigated both the copper-catalyzed and enzyme-catalyzed oxidation of ascorbic acid in a number of fruits and vegetables.

PROCEDURE AND METHODS

All vegetables were harvested between 8 and 9 a.m., taken to a nearby laboratory and triplicate samples prepared. Samples of the leafy greens (collards, kale and chard), each consisting of 100-200 g. of leaves, were packaged in perforated polyethylene bags. For the other vegetables each sample consisted of 3 specimens of the entire edible portion,

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[a] This manuscript is a contribution from the Department of Horticulture, North Carolina State College and has been approved as Paper No. 789 of the Journal Series of the Agricultural Experiment Station.

the specimens being grouped according to weight. All samples were stored on wire mesh shelves in a dark storage room having slowly circulating air under conditions of 55 ± 2° F. temperature and 75–80% relative humidity.

For the chemical analyses of vegetables a longitudinal quarter was taken from each of the 3 specimens of a sample. In the case of the leafy greens, the main rib was removed from all of the leaves and the entire sample used. The plant material was quickly shredded and mixed in a stainless steel bowl. Aliquot portions were removed and weighed into tared plastic beakers containing the appropriate extracting solutions for the ascorbic acid determinations and measurements of enzyme activity.

Reduced ascorbic acid was determined by the Morell method *(9)* as outlined in the Southern Cooperative Bulletin *(13)*. The conversion of the dehydroascorbic acid to the reduced form was accomplished as described by Rubin *et al. (14)*, and the total ascorbic acid then measured by the Morell method. The difference between the value for the total (TAA) and the reduced ascorbic acid (RAA) is indicated as the content of dehydroascorbic acid (DHA).

For measuring the ascorbic acid oxidase activity, 25 g. of the shredded plant material were blended for 3 minutes in 100 ml. of cold 0.4 M sodium chloride. The suspension was held for 2 hours at 1° C., then centrifuged for 20 minutes at 3000 r.p.m. (International centrifuge, size 1, Model SVB). The clear supernatant liquor was decanted and kept in an ice bath until used in the measurement. (This period never exceeded 20 minutes.) The reaction mixture consisted of the following: 5 ml. of a stock ascorbic acid solution containing 1250 mcg./ml. and buffered to pH 6; 35 ml. phosphate-citrate buffer at pH 6.0 and 10 ml. of the centrifuged plant extract. The reaction mixture was held at 30° C. for 15 minutes and then an aliquot was removed, diluted to a measurable concentration with 3% meta-phosphoric acid, buffered to pH 3.5 with sodium acetate, and the reduced ascorbic acid remaining in the aliquot measured spectrophotometrically using 2,6-dichlorophenolindophenol. A control mixture was used which was identical with the reaction mixture except for the substitution of 10 ml. of 0.4M sodium chloride solution for the 10 ml. of the plant extract. It is possible with the method employed that a portion of the oxidation measured was not enzymatic but due to other substances in the plant extract. However, in preliminary work using boiled extracts of squash, cabbage and collards, practically no oxidation of the ascorbic acid occurred during the reaction period.

This method is an adaptation of that employed by Huelin and Stephens in the characterization of ascorbic acid oxidase of cabbage *(6)*. They reported that the maximum activity occurred at pH 6. The solubility of the enzyme was also investigated and found to be the greatest in a 0.4 M salt solution. It is realized that these conditions of extraction and the pH of the reaction mixture may not be the optimum for the ascorbic acid oxidase activity for all of the plant material studied and that the data obtained should be considered as relative values. In this respect it has been shown that for cabbage *(6)* and squash *(19)* the optimum pH for maximum activity is approximately that of the plant suspension.

RESULTS AND DISCUSSION

Data for the ascorbic acid analyses are presented in Table 1 with the variation of the means of the total ascorbic acid analyses. The "after shelf life" data were not adjusted for the small weight loss that occurred during the storage period except in the calculation of the retention of total ascorbic acid. With the exception of cabbage and the Early Yellow Summer Crookneck variety of squash, the weight losses were less than 5% (Table 2).

The importance of including dehydroascorbic acid in determinations of the Vitamin C content of certain vegetables can be seen from inspection of the data. The different vegetables varied widely in their content of ascorbic acid, but only in the case of kohlrabi and the December harvest of collards was the dehydroascorbic acid content less than one-fourth that of the reduced ascorbic acid content for the "at harvest" values. The effect of environmental

TABLE 1

Average ascorbic acid content[1] of vegetables at harvest, and after 5 days' storage at 55°F.

Vegetable	Variety	At harvest			After shelf life			Retention of TAA
		RAA	DHA	TAA	RAA	DHA	TAA	
		mg./100 g.	*mg./100 g.*	*mg./100 g.*	*mg./100 g.*	*mg./100 g.*	*mg./100 g.*	*percent*
Kohlrabi	Early White Vienna	51.5	8.5	60.0 ± 5.68[2]	56.0	9.4	65.4 ± 1.79[2]	108.3
Collard	Hanover							
	June 12 Harvest	62.3	73.7	136.0 ± 1.47	102.7	11.8	114.5 ± 6.14	82.3
	July 2 Harvest	69.8	98.8	167.6	85.8	17.3	103.1	61.5
	Dec. Harvest	121.3	17.3	138.6	.....	.....	.....	
Chard	Lucullus	23.0	7.6	30.6 ± 2.32	7.3	30.2	37.5 ± 2.41	118.3
Kale	Vates Early Dwarf	62.8	83.7	146.5 ± 7.97	99.7	3.3	103.0 ± 1.00	68.8
Cabbage	Early Jersey Wakefield	48.1	15.3	63.4 ± 3.80	50.2	9.0	59.2 ± 0.60	87.5
Squash	Early Summer Yellow Crookneck	24.5	16.5	41.0 ± 1.00	10.9	10.2	21.1 ± 1.11	47.1
	White Bush Scallop	25.0	15.0	40.0 ± 0.0	8.5	7.1	15.6 ± 3.18	37.5
	Dark Green Zucchini	27.1	6.7	33.8 ± 0.23	5.2	8.3	13.5 ± 2.02	38.2

[1] Data are the average of 3 samples each with the exception of December harvest of collards which is the average of 6 samples. Values are expressed on the fresh weight basis. Percent retention is calculated on the TAA content of stored vegetables adjusted to an "at harvest" basis.

[2] Standard deviation of the means of triplicate samples. This value for RAA and DHA is approximately the same order of magnitude.

TABLE 2

Weight loss of vegetables during shelf life

Vegetable	Variety	Days of storage	Percent weight loss	
			Average[2]	Range
Kohlrabi	Early White Vienna	4[1]	0.73	0.52 – 0.89
Collard	Hanover	6[1]	2.26	1.98 – 2.50
Swiss Chard	Lucullus	4[1]	3.47	1.96 – 4.30
Kale	Vates Early Dwarf	6	2.67	2.40 – 3.20
Cabbage	Early Jersey Wakefield	6	6.06	5.28 – 6.09
Squash	Early Yellow Summer Crookneck	6	8.08	6.15 – 9.83
	White Bush Scallop	6	3.73	3.08 – 4.17
	Dark Green Zucchini	6	4.73	2.34 – 6.10

[1] Stored in perforated polyethylene bags at 55° F. All other vegetables stored in slowly circulating air with a relative humidity of 75–80% and at a temperature of 55° F.

[2] Average of three replicates with 3 samples each or a total of 9 observations. In the case of the leafy greens, this value is the average of 3 samples.

factors, presumably temperature, upon the ascorbic acid content of the collards is evident, for although the total ascorbic acid content of the summer and fall-harvested collards is in the same range, a much greater proportion of the ascorbic acid is in the reduced form in the material harvested in the fall. A similar relationship is found also in a comparison of the before and after shelf life data of the summer-harvested collards and kale. It is probable that data from analyses of fall-harvested kale would be similar to that of the fall-harvested collards since these two vegetables are of the same botanical subspecies. Collards and kale are not grown commercially during the warm summer months and accordingly the data for the fall-harvested collards should be taken as the more representative of this crop.

It is apparent from the "after shelf life" data that not all of the vegetables responded to storage in the same manner. Little or no loss of total ascorbic acid occurred in cabbage, chard, or kohlrabi. (The increase apparent in chard and kohlrabi may be due to sample variation.) In the case of chard, there was a considerable increase in the content of the dehydro form during the 5 day shelf life and a decrease in the content of the reduced form. Zepplin and Elvehjem *(22)* reported a decrease in the dehydroascorbic acid content of chard held for 3 days in crushed ice, but no loss of total ascorbic acid. They did, however, find an increase in the dehydroascorbic acid content of snap beans after 3 days' storage in crushed ice. In collards and kale the reverse situation existed, with a sizeable increase occurring in the reduced form, accompanied by a disproportionate decrease in the dehydro form. This resulted in a loss in the overall total ascorbic acid content during the shelf life. One of the characteristics of ascorbic acid is its reversible oxidation to dehydroascorbic acid, and it seems probable that the increase in the reduced form in the stored collards and kale is from the transformation of the dehydro form. Some synthesis of ascorbic acid may have taken place in the leaves during storage. It is interesting to note the similarity of the "after shelf life" data for the summer-harvested collards and the December "at harvest" data. This is further indication that temperature may be a factor in the equilibrium between reduced and dehydroascorbic acid. In the squash a loss of approximately 50 to 60% of the total ascorbic acid occurred. In the variety Dark Green Zucchini this loss was due entirely to a decrease in the reduced ascorbic

acid content. The "after shelf life" values obtained for the Yellow Crookneck variety of squash are similar to those reported by Worthington *(21)* for this same variety when samples were procured from a local store. For cabbage and collards, the inclusion of dehydroascorbic acid in the vitamin C determination resulted in a greater loss of this vitamin during a short holding period than has previously been reported.

In a review of studies on cabbage by Patton and Greene *(11)*, it is pointed out that some workers found no loss of vitamin C, even after two months of storage. In a study comparable to the present investigation the average loss of reduced ascorbic acid for 3 strains of cabbage held one week at 4 to 5° C. was approximately 5%. It is indicated from previous work on collards that losses of approximately 10% of the reduced acid content may be expected during short holding periods, particularly if some refrigeration is used *(15)*.

The ascorbic acid oxidase activity of extracts from the various vegetables is shown in Table 3. The high activity found in the squash is in agreement

TABLE 3

Ascorbic acid oxidase activity of vegetables

Vegetable	Variety	Percent oxidation in 15 minutes	
		Average[1]	Range
Kohlrabi	Early White Vienna	34.0	30 – 33.6
Collard	Hanover June 12	70.7	70.4 – 70.8
	Dec. 14	30.8	28.9 – 32.7
Swiss Chard	Lucullus	14.7	9.6 – 20.0
Kale	Vates Early Dwarf	82.5	78.4 – 84.8
Cabbage	Early Jersey Wakefield	53.5	52.8 – 54.8
Squash	Early Yellow Summer Crookneck	90.5	82.4 – 99.2
	White Bush Scallop	87.3	80.0 – 92.0
	Dark Green Zucchini	94.8	91.2 – 98.4

[1] Average value from 3 samples, each sample consisting of a composite of 3 vegetables, except in the case of the leafy greens, Collard, Kale, and Chard where 100–200 g. of leaves comprised a sample.

with previous reports *(19)*. The difference in the activity of the extracts from collards of different harvest dates is not altogether unexpected. The December-harvested plants had been subjected to much lower temperatures and should have been in a much lower state of physiological activity than the summer-harvested plants. Inconsistencies are apparent if an attempt is made to relate the ascorbic acid oxidase activity of the vegetables to the loss in storage of the substrate, reduced ascorbic acid. However, the ascorbic acid oxidase activity as determined for the plant extracts would undoubtedly be higher than the activity of the enzyme in the vegetables at the 55° F. shelf life temperature. Squash, with the highest activity, and chard, with the lowest activity, were the only vegetables in which a loss of reduced ascorbic acid occurred during the storage period. There is a possibility of copper-catalyzed or indirect oxidation taking place in the chard. However, even with a low rate of activity the 4-day storage period should presumably have provided ample time for the enzymatic conversion of the reduced to the dehydro form. Of perhaps more significance is the apparent accumulation of dehydroascorbic acid in the chard, for it is only when degradation has proceeded beyond this form that the vitamin C value of the plant material is lost.

SUMMARY

The content of reduced, dehydro, and total ascorbic acid was determined for a number of vegetables at harvest, and after a simulated shelf life. In addition the ascorbic acid oxidase activity of the vegetables at harvest was determined.

Appreciable amounts of dehydroascorbic acid were found in the vegetables at harvest, and only in two cases was the content of dehydroascorbic acid less than one-fourth the content of reduced ascorbic acid. Considerable difference in the proportion of these two forms of ascorbic acid was observed in collards harvested at different seasons and also in collards and kale before and after storage. An increase in the content of dehydroascorbic acid of chard occurred during the shelf life.

A loss of total ascorbic acid during the shelf life occurred with all of the vegetables except chard and kohlrabi.

Ascorbic acid oxidase activity was found in all of the vegetables analyzed. The highest activity was found in squash and the least in kohlrabi and chard.

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THE EFFECT OF RADIATION PROCESSING UPON THE *IN VITRO* DIGESTIBILITY AND NUTRITIONAL QUALITY OF PROTEINS

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The potential use of gamma radiations for the preservation of food makes it important to determine the effect of this type of treatment upon the nutritional quality of food proteins. In this respect it has been shown that although moderate doses of ionizing radiations produce significant destruction of individual amino acids in pure solution *(3, 4, 7, 13)*, proteins must be irradiated at several times the usual sterilization dose range of 0.5 to 6.0 million rep before nutritionally significant losses of amino acids are observed *(1, 8)*. However, viscosity and solubility studies indicated that irradiation produces definite increases in cross linking as well as degradation of the treated proteins *(1)*. Since these structural changes can modify the susceptibility of the treated proteins to digestion it became of interest to study the *in vitro* digestibility of irradiated proteins and to estimate the effects of digestibility changes upon nutritional value. Recently, the relationship between the pattern of amino acids released by digestive enzymes and the biological value of food proteins was studied in this laboratory. The pattern of amino acids released *in vitro* by pepsin revealed differences between proteins which were not apparent from their total essential amino acid content nor from the patterns existing when the pepsin digests were further digested with trypsin and erepsin. As a consequence of these results, an amino acid index was devised which took into account the availability of amino acids during *in vitro* pepsin digestion. The new index combined the pattern of essential amino acids released by *in vitro* pepsin digestion with the amino acid pattern of the remainder of the protein to produce an integrated index—the Pepsin Digest-Residue (PDR) Amino Acid Index. The PDR index was closely correlated with the net protein utilization value (biological value x digestibility) of a variety of proteins *(12)*. Subsequent application of the new procedure to heat-processed protein foods indicated that the PDR index also accurately predicted changes in the net utilization of the treated proteins *(11)*.

The purpose of the present study was (a) to determine the patterns of amino acids released by *in vitro* enzymatic digestion of representative food proteins subjected to ionizing radiation and (b) to compare the nutritional quality of the irradiated and heat processed proteins as measured by their PDR index values.

METHODS AND MATERIALS

Procedures for preparation of the enzymatic digests, microbiological assay for amino acids, and calculation of the PDR index have been previously described *(12)*.

Evaporated milk was prepared at the Pet Milk Company, New Glarus, Wis. by the usual production procedure. In this procedure the raw milk is heated for 10 minutes at 210° F. and then condensed in a double-effect evaporator. For the first effect the milk

is treated at 180° F. for 2½ minutes; in the second effect the milk is further treated at 125° F. for 2½ minutes. Following canning of the unsterilized condensed milk, samples for control purposes and for subsequent irradiation were removed from the conveyor belt. Remaining samples were heat-sterilized at 242° F. for 14 minutes. All canned condensed milk samples were kept chilled until analyzed. Freezing of milk samples was avoided to minimize structural changes in the proteins.

Beef[a] used in this study was obtained from the *longissimus dorsi* muscles of premium grade steers. Meat was trimmed, 20% fat by weight was added, and ground through a 1 inch by 1½ inch plate and then a ¼ inch plate. Pork meat was obtained from the *longissimus dorsi* muscles of matched pork loins. After being trimmed to 20% trimmable fat, the pork meat was ground in the same manner as the beef. Samples were canned under 20 inches vacuum in No. 2½ cans. Heat processed samples were autoclaved at 240° F. for 161 minutes (FO = 6.95). Unsterilized samples prepared for irradiation were stored frozen.

Turkeys used in this study were all of the same brood and fed in the same flock. Meat was removed in large pieces and excess fat removed. Both dark and light meat and skin were cut into strips, ground, and mixed. Samples were vacuum packed in No. 2½ cans. Heat processing was performed at 240° F. for 114 minutes (FO = 11.6). Unsterilized samples prepared for irradiation were kept frozen. All meat samples were stored at −20° F. after processing until analyzed.

Irradiation sterilization was done by gamma irradiation from spent fuel rods at the Argonne National Laboratory.[b] All samples were given radiation doses of 2 million rep as determined by ferrous dosimetry. Milk samples were held at approximately 35° F. and meat samples were kept frozen in solid CO_2 packs until placed in the irradiation canal. Since the longest period of irradiation was approximately 2 hours, the meat samples were probably frozen throughout the treatment. Previous tests had indicated that aqueous solutions in No. 2 cans remain frozen for 3 hours in the canal.

RESULTS

Irradiation with 2 x 10^6 rep gamma rays produced no significant destruction of amino acids in milk, turkey, or beef protein. In pork there was a 16% loss of cystine following irradiation, but no other destruction was observed. Following heat processing, no loss of amino acids was found in any of the protein foods studied.

Rate of release of amino acids during pepsin digestion was approximately the same for both irradiated and non-irradiated turkey meat (Table 1). In the case of irradiated evaporated milk the liberation of cystine was reduced 29%, but the remaining amino acids were unaffected. Similarly, with irradiated pork, only phenylalanine was not as readily released by pepsin, and for beef only phenylalanine and tryptophan were reduced. On the other hand, there was a decided decrease in the release by pepsin of all the amino acids measured in the heat-processed meat samples. For heat-processed evaporated milk, only the rate of release of cystine was reduced.

Incubation of the 4 protein foods with pepsin and pancreatin in succession also indicated that irradiation with 2 x 10^6 rep did not produce marked changes in the susceptibility of the proteins to enzymatic digestion (Table 2). In evaporated milk the release of all the amino acids studied except cystine was not affected by irradiation; the liberation of cystine was 57% less in the irradiated milk digests than in the unprocessed samples. In the pepsin plus pancreatin digests of irradiated beef protein, only the quantity of histidine was reduced; and for turkey and pork no differences were noted between the irradiated and unprocessed samples.

Contrary to the limited effect of irradiation, heat processing markedly decreased the susceptibility of the 3 meat samples to the combined action of pepsin and pancreatin. However, in the case of evaporated milk similar to the results obtained with irradiation,

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[a] Beef, pork, and turkey samples were prepared in the Animal Products Branch, Quartermaster Food and Container Institute for the Armed Forces under the direction of Mr. R. Buscemi.

[b] The samples were irradiated under the direction of Miss H. Gladys Swope, in charge of the Gamma Radiation Facility, Argonne National Laboratories.

TABLE 1

The concentrations of amino acids in pepsin digests of food proteins subjected to irradiation and thermal processing

	Evaporated Milk			Turkey			Pork			Beef		
	Con.[1]	Irr.[2] mg/g	Therm.[3]	Con.	Irr. mg/g	Therm.	Con.	Irr. mg/g	Therm.	Con.	Irr. mg/g	Therm.
Methionine	1.2	1.0	1.2	6.9	6.8	4.8	10.0	10.0	6.6	10.0	9.3	7.0
Cystine	0.17	0.12	0.15	1.04	0.99	0.52	1.4	1.1	0.44	1.1	1.1	0.32
Phenylalanine	5.7	5.2	6.2	8.9	8.1	4.8	10.0	9.1	4.7	11.6	9.5	5.1
Tyrosine	5.5	5.4	5.9	5.5	5.4	3.1	5.6	5.4	3.0	6.5	5.7	3.1
Histidine	0.26	0.22	0.26	1.7	1.6	1.2	1.6	1.5	1.1	2.6	2.3	2.1
Lysine	0.60	0.70	0.74	2.8	2.6	2.1	3.2	2.4	1.8	3.4	3.1	1.9
Tryptophan	4.5	4.2	4.7	4.2	3.6	1.5	5.2	4.6	2.0	7.0	5.3	2.1
Leucine	39.0	37.0	41.7	46.9	47.5	30.4	48.5	46.1	27.2	62.2	59.0	38.2
Isoleucine	8.9	7.8	7.8	34.3	35.0	31.5	25.4	24.8	14.0	29.2	30.1	14.8
Valine	2.5	2.4	2.7	10.6	10.8	6.8	11.5	10.6	6.9	12.4	11.4	7.0
Threonine	13.8	13.4	14.4	31.0	28.0	21.2	29.0	26.9	16.5	27.1	27.4	17.5

[1] Unprocessed controls.
[2] Irradiated with 2 x 10^6 rep.
[3] Thermal processed (see Methods).

TABLE 2

The concentrations of amino acids in pepsin plus pancreatin digests of food proteins subjected to irradiation and thermal processing

	Evaporated Milk			Turkey			Pork			Beef		
	Con.[1]	Irr.[2] mg/g	Therm.[3]	Con.	Irr. mg/g	Therm.	Con.	Irr. mg/g	Therm.	Con.	Irr. mg/g	Therm.
Methionine	12.8	11.8	12.6	19.7	19.4	16.2	22.5	23.7	17.4	21.0	20.4	17.8
Cystine	1.4	0.6	0.9	4.3	4.6	2.8	6.5	5.9	3.3	6.6	5.9	2.8
Phenylalanine	22.6	22.5	23.9	29.5	28.1	23.2	30.8	34.4	21.9	28.3	29.8	23.4
Tyrosine	27.5	25.0	28.7	28.9	28.5	23.9	29.7	28.0	21.9	27.6	28.4	23.5
Histidine	14.2	13.2	12.2	18.0	16.6	14.4	14.5	14.4	10.4	19.3	14.4	16.2
Lysine	61.6	60.3	57.5	82.6	76.8	68.6	83.9	81.8	63.3	81.2	82.0	68.2
Tryptophan	10.8	9.1	10.4	10.0	9.7	8.0	10.8	12.6	8.1	10.6	10.1	8.1
Leucine	81.5	74.6	80.5	69.4	72.1	59.2	71.0	70.8	55.5	83.4	69.2	76.3
Isoleucine	37.5	37.0	39.8	52.4	48.9	43.8	38.6	40.4	32.2	42.8	40.7	41.0
Valine	22.7	23.0	21.6	37.7	38.0	30.5	41.0	40.4	30.6	40.6	40.6	31.6
Threonine	34.7	31.8	35.4	45.8	41.1	38.5	41.7	52.0	36.0	42.7	42.8	37.2

[1] Unprocessed controls.
[2] Irradiated with 2×10^6 rep.
[3] Thermal processed (see Methods).

the liberation of only cystine was reduced following heat processing. It should be noted that the heat processing time for evaporated milk is much shorter than that necessary for meat samples.

PDR amino acid indexes of net protein utilization (biological value x digestibility) for the 4 proteins studied in this series of experiments are presented in Table 3. In each

TABLE 3

The PDR index of net protein utilization of foods subjected to thermal and radiation sterilization

Protein	PDR Index[1]		
	Unprocessed	Thermal Processed	Irradiated
Beef	86	85	87
Pork	88	80	89
Turkey	74	71	74
Evap. milk	75	75	74

[1] Standard errors; beef 0.76, pork 1.29, turkey 0.92, and milk 0.83.

case, irradiation with 2×10^6 rep produced no significant change in nutritional quality as measured by the PDR index. Heat processing produced an apparent but not significant (5% level) decrease in the PDR value of turkey and a very significant lowering in the PDR index of pork protein.

DISCUSSION

The biological value of evaporated milk has been reported to be significantly lowered by irradiation with 3×10^6 rep gamma rays *(5)*. No change in digestibility was observed. These results were interpreted as suggesting that some essential amino acid was partially destroyed or bound so as not to be available at an optimum rate for most efficient utilization. In subsequent reports from the same laboratory *(6, 9)* it was shown that cystine and methionine were destroyed to the extent of 43.7% and 29.4% respectively by 3×10^6 rep. They also found that the effects of irradiation upon the biological value of the milk proteins could be compensated for by supplementation of the irradiated milk with 0.3% cystine. Nevertheless, the data reported in the present study indicated that irradiation with 2×10^6 rep does not significantly destroy amino acids in evaporated milk. Also, with the exception of cystine there was no significant change in the enzymatic availability of amino acids in the irradiated milk proteins. Diminished enzymatic liberation of cystine by itself would not be expected to have nutritional significance since the concentration of cystine in milk proteins is very low, especially in comparison with that of methionine. However, calculation of the PDR index of irradiated milk using methionine and cystine values which reflect the destruction reported by Rama Rao and Johnson *(9)* produces a decrease of 4.5% in the PDR index which is similar to the 5.0% decrease in biological value found by Metta and Johnson in their later experiments *(6)*. Results thus suggest that gamma irradiation with up to 2×10^6 rep does not cause significant destruction or change in availability of amino acids in the proteins of evaporated milk; however, with larger doses some destruction of cystine and methionine may occur which depresses the nutritional value of the irradiated milk.

In the case of turkey, pork, or beef, irradiation with 2 x 10^6 rep caused only minor alterations in the concentration or enzymatic availability of amino acids. The PDR indices of these protein foods were therefore not significantly changed. In this respect, Metta and Johnson *(5)* reported no change in the biological value of beef following irradiation with 3 x 10^6 rep and Calloway *et al. (2)* obtained similar results with turkey irradiated up to 6 x 10^6 rep. Also, Rosner *(10)* found no amino acid destruction or change in the pepsin digestibility of beef irradiated up to 6 x 10^6 rep.

Heat-treated proteins of turkey, pork, and beef did not show evidence of amino acid destruction nor of changes in the pattern of amino acids released by digestive enzymes. However, the heat treatment did cause a significant decrease in the quantity of amino acids released by *in vitro* incubation with pepsin. It is evident from previous work *(11)* that diminished susceptibility to *in vitro* pepsin digestion usually results in a decrease in the nutritional quality of a protein. The observed decrease in pepsin digestibility of heated pork and turkey, were thus reflected in lower PDR index values, although only for pork was the change statistically significant. However, whereas heated beef protein was less susceptible to pepsin digestion, the PDR index value reported here and the biological value as obtained by the Mitchell method of nitrogen balance *(5)* were not decreased. Inspection of the data for calculation of the PDR index indicated that the heated beef had a higher essential amino acid content per unit of nitrogen than did the untreated beef. This phenomenon, which may be due to volatilization of non-essential nitrogenous compounds, is possibly one reason that heat-processed beef protein appears to retain completely its nutritional value on a nitrogen basis, in spite of the fact that the protein is considerably less susceptible to enzymatic digestion. A more important reason is related to the fact that following treatment with pepsin the amino acid pattern in the undigested fraction of heated beef proteins is superior (relative to the standard egg protein) to the pattern of the same residual fraction of raw beef protein. This improvement in the amino acid pattern of protein not digested by pepsin but subsequently digested by intestinal enzymes probably compensates for the effect of decreased pepsin susceptibility of the heat processed beef proteins.

SUMMARY

Representative protein foods were subjected to 2 x 10^6 rep gamma rays from spent fuel rods and to thermal processing.

Irradiation produced no significant destruction of essential amino acids in milk, turkey, or beef protein. In pork, no destruction of amino acids was observed except for a small loss of cystine. Following heat processing no loss of amino acids was found in any of the protein foods studied.

Irradiation resulted in substantially no change in the enzymatic availability of amino acids, whereas thermal processing caused significant decreases in the digestibility of the proteins studied.

No significant changes in the protein quality, as measured by the PDR index, occurred from irradiation. Heat processing produced a small but not significant decrease in the PDR index of turkey and a very significant lowering in the PDR index of pork protein.

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A MICRO-METHOD AND APPARATUS FOR THE DETERMINATION OF RATES OF DESTRUCTION OF BACTERIAL SPORES SUBJECTED TO HEAT AND BACTERICIDAL AGENTS [a]

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A procedure and the necessary apparatus have been devised in an effort to simplify the determination of rates of destruction of bacterial spores subjected to heat up to temperatures of approximately 121° C. (250° F.). There are many reports in the literature dealing with the problem of subjecting bacterial spores to heat to study their rate of destruction. Stumbo's thermoresistometer *(4)*, and the apparatus of Pflug and Esselen *(1)* are two outstanding instruments for this purpose and are particularly useful for the study of destruction rates of bacterial spores subjected to high temperature-short time processing. These methods use open cups introduced into and out of steam chambers under pressure. Stern and Proctor *(3)* described a micro-method for the multiple determination of rates of destruction of bacteria and bacterial spores subjected to heat in which the organisms were carried in capillary tubes and subcultured.

The work represented in this report was undertaken in an effort to simplify the method of using glass capillary tubes. The apparatus consists of two parts: a device for accurately dispensing a desired volume of material into the capillary tubes, and an apparatus for evacuating the tubes and introducing culture media into the tubes after heating. The latter permits incubating the capillary tubes directly.

EXPERIMENTAL

Apparatus. The apparatus for filling capillary tubes consists of a 1.00 cc. tuberculin syringe mounted in an aluminum holder [b] with a micrometer head [c] attached (Figure 1). By choosing a tuberculin syringe calibrated and marked so that the distance between 0 and 1.0 cc. equals 2.5 inches, one revolution of the micrometer head will move the syringe to deliver exactly 0.01 cc. A 2-inch, 20-gauge, stainless steel hypodermic needle is used in conjunction with the syringe. In use, the syringe holder is mounted on a ring stand with a clamp to permit manipulation with both hands.

The vacuum filler is illustrated in Figure 2 and diagrammed in Figure 3. It consists principally of a 3-way double oblique bore stopcock and a packing gland (Conax No. MPG [d]) to hold tightly a capillary tube. Media reservoir and vacuum line from a water aspirator are attached to the stopcock. Tubing and gasket in the packing gland are of surgical rubber to withstand autoclaving. Apparatus is mounted on a shortened stand by one clamp. For sterilization, a folded cloth is wrapped over the packing gland, apparatus is lowered on the support rod, and complete unit with stand is placed in the

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[a] Technical Paper No. 1040, Oregon Agricultural Experiment Station. Contribution of the Department of Food Technology.

[b] Designed and constructed by Mr. C. J. Filz, Mechanician, Physics Dept., Oregon State College.

[c] No. 263, 1-inch graduated in thousandths. The L. S. Starrett Co., Athol, Mass.

[d] Conax Corporation, 7811 Sheridan Drive, Buffalo 21, New York.

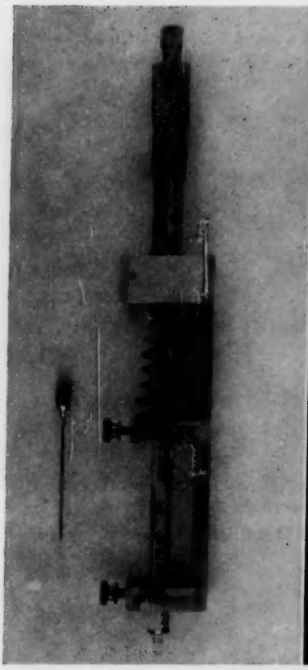


Figure 1. Micro-volumetric dispensing device.

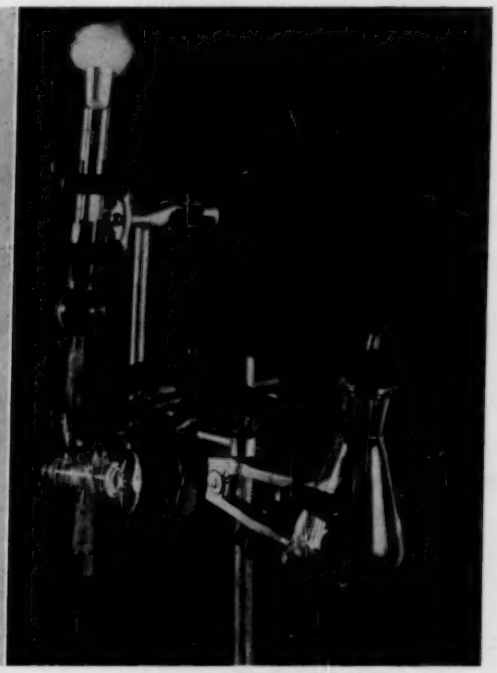


Figure 2. Vacuum filler.

autoclave. After sterilization, apparatus is raised on the stand to operating height, reservoir is filled with sterile media, cloth is removed from the packing gland, and vacuum line is attached to the water aspirator.

In operation, the sterile opened end of a capillary tube is inserted in packing gland as shown in Figure 3. Screw cap is tightened with the fingers and vacuum line is opened by the 3-way stopcock and left in this position for approximately 3 seconds. Stopcock is then turned to admit media into evacuated tube. Media flows into the tube in a fraction of a second. Stopcock is then turned horizontally, packing gland is loosened, and capillary tube is removed. The apparatus is ready for the next capillary tube to be inserted. The capillary tubes used had inside diameters of 0.8 to 1.1 mm., a wall thickness approximately 0.25 mm., and were 68 mm. in length.

The oil bath employed in this work consisted of a 3-gallon aluminum bucket mounted inside a wooden tub with approximately 2 inches of insulation between the walls of the 2 containers. Petroleum jelly was maintained at desired temperature by a thermoregulator, electric heating coils, and electric stirrer.

The test organism. The test organism used was PA 3679 obtained from the National Canners Association, Berkeley, California. Spores in large concentration were produced by growing the organism in the liver broth medium used by Stumbo, Murphy, and Cochran *(5)*. Spore concentration, estimated by the modified method of Wynne, Schmieding and Daye *(6)*, was found to be 3×10^8 spores per ml.

Preparation and processing of samples. The material subjected to heat treatment consisted of meat, spores, and antibiotic. This was made up so that 0.01 ml. contained 0.005 ml. meat, 10,000 spores and a solution of antibiotic of the desired concentration. Antibiotics used were PA 122, neomycin, and oxamycin. Filling was planned to give a final meat volume of one half of the total volume.

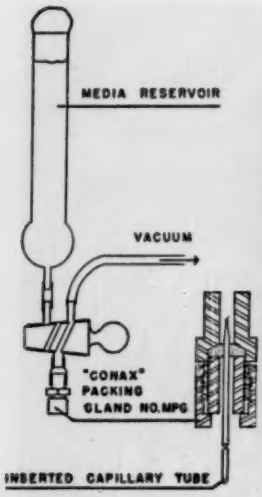
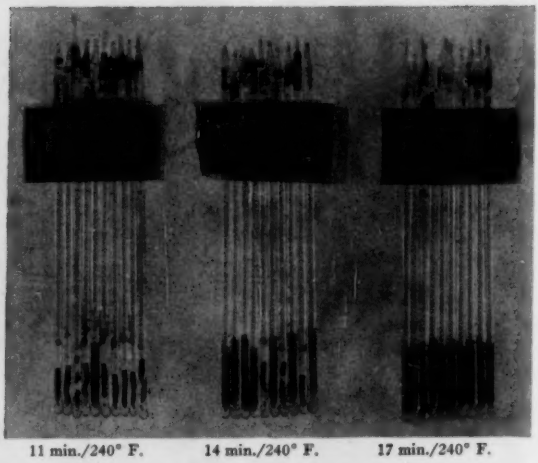


Figure 3. Vacuum filling apparatus.



11 min./240° F. 14 min./240° F. 17 min./240° F.

Figure 4. Inoculated tubes.

A typical dilution was as follows:

Meat	10 ml.
Spore suspension	6.7 ml.
Antibiotic	0.5 ml. (stock 400 p.p.m.)
Sterile distilled water	2.8 ml.
Total	20.0 ml. (10 p.p.m. antibiotic)

Whole lean beef was ground twice and then pressed through several layers of muslin in a laboratory hydraulic press. This resulted in a meat of such consistency that it was easily dispensed through a 20-gauge hypodermic needle.

Meat, PA 3679 spores (10,000 per 0.01 ml. of the final mixture), and antibiotic were slurried and aliquots drawn into the sterile syringe which had been mounted in the holder (Figure 1); 0.01 cc. of meat slurry was put into each of the sterile capillary tubes that had been sealed at one end. The open end of each tube was then sealed in a small gas flame. Approximately 20 filled and sealed tubes at a time were placed in a cotton padded 15 ml. centrifuge tube and centrifuged for 30 seconds at 1,000 r.p.m. or sufficiently to bring the cylinders of meat slurry in the capillary tubes down to one end. It is important that no air bubbles remain in the meat slurry in the capillary tubes. During the entire operation, care was taken to keep the meat slurry-spore mixtures and the filled capillary tubes in a refrigerator when not actually being handled.

For heat treatment, filled capillary tubes were removed from the refrigerator, mounted in groups of 10 in a partially extended spiral spring holder, warmed to room temperature, and completely submerged in the oil bath at designated temperature and time. A stopwatch was used to time heat treatments. At the end of the designated times, tubes were removed from oil bath and plunged into cold water. Heating and cooling lag times have been estimated to be 5 seconds each at 115.6° C. (240° F.). Tests were conducted at a temperature of 115.6° C. (240° F.) and times of 14, 17, 20 and 25 minutes.

After cooling, capillary tubes were washed in solvent to remove congealed oil, placed in cleaning solution for 10 minutes, and finally rinsed in sterile distilled water to remove any traces of cleaning solution.

Ten replicate capillary tubes of each time-temperature-antibiotic level were evaluated by culturing in 15 x 150 mm. screw cap culture tubes. Under aseptic conditions, each of

the 10 capillary tubes was placed in an individual culture tube to which 5 ml. of sterile distilled water had been added. Capillary tubes were handled with sterile forceps through out. Each capillary tube was broken and well crushed in its respective culture tube. Approximately 15 ml. of sterile, warm media were added and contents mixed well by rolling the tube betwen the hands. Medium used was that of Wynne, Schmieding, and Daye *(6)*. After the medium solidified, approximately ½ inch of sterile capping mixture was layered on the solid medium in each tube. Capping mixture was a 1:1:4 mixture of petroleum jelly, paraffin, and mineral oil. Culture tubes were incubated at 29.4° C. (85° F.).

An identical set of 10 replicate tubes, after cleaning, were carefully heated in a small flame at the top, drawn out to a fine diameter and broken off aseptically, thereby opening the tube and sterilizing the opened end in the same operation. These tubes were then filled with the same medium (except without agar) employed in the large culture tubes, using the vacuum filling apparatus previously described. After filling, the open end of each tube was sealed with household cement and tubes were incubated at 29.4° C. (86° F.) after being taped together as illustrated in Figure 4.

RESULTS

Four runs were completed to test the apparatus and procedure. Capillary tubes were evaluated on the basis of changes in color and appearance of the meat slurry during incubation. Meat in those tubes in which viable spores were present became dark and noticeably shrunken on incubation for approximately 60 days as shown in Figure 4. Meat in tubes showing no such change was presumed to be sterile. Tubes of the first set handled according to the new procedure were opened, and stained preparations of the contents made to determine the presence or absence of vegetative cells. In all cases, presence of vegetative cells was associated with changes in color and appearance, while tubes showing no change showed no vegetative cells.

From the number of positive and negative tubes in each set, D values were obtained by treating the data according to the method described by Stumbo, Murphy, and Cochran *(5)*. D is defined as the time required to reduce the number of viable bacteria 90% at any given temperature as originally suggested by Schmidt *(2)*. Calculated average D_{240} values appear in Tables 1 and 2. These may be compared with average D_{240} values obtained by other workers as shown in Table 3. The values reported here show good agreement with other data.

TABLE 1

Average D_{240} values using micro method by which the spores were incubated directly in the capillary tubes

Antibiotic	p.p.m.	Average D_{240}
None	....	4.33
PA 122	10	4.13
PA 122	50	4.68
Neomycin	10	3.67
Neomycin	50	3.45
Oxamycin	10	4.04
Oxamycin	50	4.11

TABLE 2

Average D_{240} values using the crushed tube method of Stern and Proctor (3)

Antibiotics	p.p.m.	Average D_{240}
None	....	5.37
Neomycin	10	4.39
Neomycin	50	4.45

TABLE 3

Reported average D_{240} values

D_{240}	Substrate	Reference
4.17	M/15 Neutral Phosphate Buffer	*(1)*
5.21	Pureed Peas	*(5)*
4.64	Cooked Fresh Pork	*(5)*

The standard tubes (capillary tubes crushed in 15 x 150 mm. culture) were evaluated by visual inspection, i.e., presence or absence of obvious colonies in the culture tubes and/or presence of gas. D values are calculated for these data by the method indicated above and are recorded in Table 2.

DISCUSSION

The new micro-method of filling the treated capillary tubes with media and incubating them directly means a great saving in media usage, incubator space, and elimination of much manipulation that might introduce contamination. Incubated capillary tubes are easy to handle and detection of positive tubes is readily apparent. The technique of handling the apparatus is not difficult to learn. Effect of the added media on top of the meat slurry in the capillary tube is an unknown factor as far as adding nutrients to the meat containing the heated spores. Very little mixing takes place even when the tubes are severely vibrated by holding them against a revolving Waring blender stem. Perhaps the greatest aid to germination by the addition of media in the vacuum apparatus is the quick establishment of anaerobic conditions.

In this method bacterial spores are incubated in the media in which they were treated (meat) in the presence of the bactericidal agent being tested. This is a method to test a combination of the killing power and sustained repressant power of a bactericidal agent in low concentrations after being subjected to high temperatures.

The average D_{240} values obtained from the vacuum filling method and those obtained by subculturing where the added antibiotic would be diluted out are in good agreement. Also the average D_{240} values agree with those of other workers. Of the three antibiotics tested, PA 122 (an experimental antibiotic), neomycin and oxamycin, none have shown conclusive effect in the reduction of the D_{240} value in the presence of heat as used in this experimentation.

SUMMARY

Experiments were carried out in an effort to test the validity of a new micro-method and apparatus for determining rates of destruction of bacterial spores subjected to heat and bactericidal agents. The method of admitting the experimental sample in the capillary tubes and the evacuation and addition of media after heat processing is described in detail. A study of the D_{240} values obtained indicates that the method results in valid data.

This method has the advantage of allowing large numbers of samples to be handled with a minimum of time and materials. Certain aspects of heat processing such as come-up time and cooling time are minimized by using capillary tubes.

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ASSAY AND PROPERTIES OF COMMERCIAL FICIN

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Proteolytic enzymes of plant origin have been used quite extensively in industry during recent years. Papain, the latex of the green fruit of *Carica papaya*, and bromelin, the papainase of pineapple juice, have been used to clarify beverages and to tenderize meats. Most recently proteolytic enzymes derived from other sources such as the latex of the fig tree, bacteria, and fungi have been used to broaden the range of industrial usage. Rhozyme P-11 (Rohm and Ha Co.) is a potent proteolytic enzyme of fungal origin *(11)*. Ficin, the proteolytic component of fig latex, has been found to tenderize meats *(10, 17)*, and Wang *(17)* concluded it to be superior to papain and bromelin for this purpose.

Whereas the properties of papain and bromelin have been quite thoroughly studied, relatively little is known about the properties of ficin. It is usually stated that papain and ficin are similar in properties. The known properties of ficin are summarized by Greenberg and Winnick *(6)*. The present work was undertaken because of the potential uses of ficin as a meat tenderizer and clarifier of beverages and the scarcity of information on this enzyme. The aim of this research is to develop a simple method for determining the proteolytic activity of ficin, to define the conditions under which this method works, and to determine some of the properties of ficin using this method.

EXPERIMENTAL

Proteolytic enzymes. Two samples of commercial ficin were tested. They were obtained from the Mann Research Laboratories, Inc., New York, N. Y., and the Nutritional Biochemical Corporation, Cleveland, Ohio. They were found to be similar in properties, and the data reported here were obtained with ficin from the latter source. The papain used was from Difco Laboratories, Inc., Detroit, Michigan, and the bromelin was from the Pineapple Research Institute of Hawaii. Enzyme solutions were prepared by dissolving a specified weight of the dry material in distilled water just before use. The protein concentration was checked by measuring the absorption at 280 mμ and all solutions of ficin were adjusted to the same concentration level. The samples of ficin were readily soluble in water, but bromelin and papain were not completely soluble.

Gelatin. Gelatin was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. It had a moisture content of 10.18% when dried at 105° C. for 24 hours and an ash content of 1.46% when ashed at 555° C. for 16 hours. It had 18.0% nitrogen by the micro-Kjeldahl method. The gelatin solutions were prepared by dissolving 5 g. of gelatin in 100 ml. of water at 75° C. After cooling, the gelatin solution was diluted with the desired buffer to give a final buffer concentration of 0.2 M and the desired concentration of gelatin. The final pH was determined accurately using a Beckman Model G pH meter. This solution was allowed to stand one hour at the temperature to be used in the experiment before it was used. Successive viscosity readings taken after this period were reproducible. Gelatin solutions increase in viscosity with age at different rates depending upon concentration of gelatin, hydrogen ion concentration, the kind of gelatin, and heat

treatment during the dissolving process *(4)*. These should be controlled in preparing solutions for viscosimetric studies.

Determination of activity. The rate of digestion of gelatin was followed by 2 methods, the modified Sorenson formol titration method of Dunn and Loshakoff *(5)*, and a viscosimetric method. Viscosity was measured using an Ostwald viscosimeter with a flow time of 78.8 seconds for water at 30° C. and was calibrated by the method of Sheeley *(15)* using aqueous glycerol solutions. A 30% glycerol solution (w/w) at 30° C. had a flow time of 184.6 seconds. It was found that the kinetic factor could be ignored in calculating the relative viscosity.

The desired amount of ficin was activated with sufficient cysteine at pH 7.0 to give a final cysteine concentration in the reaction mixture of 0.025 M. Ficin must be activated prior to beginning the measurement of the rate of decrease in viscosity. Otherwise, a period of less than maximum activity results initially. Two to 5 minutes at the temperature of the determination results in complete activation of ficin by cysteine. If sodium cyanide is used as the activator, the time must be increased. After activation, the buffered gelatin solution, at the temperature of the determination, is added to the ficin and an electric timer (reading to 0.1 sec.) is started immediately. Ten ml. of this well-mixed solution is then transferred to the viscosimeter (at the temperature of the determination). Outflow time of the mixture is measured at approximately 5-minute intervals for 40 minutes using a second electric timer. Viscosities were determined in a constant temperature water bath maintained at $\pm 0.1°$ of the desired temperature. Since outflow time cannot be neglected as compared with duration of the reaction, the moment of start of the viscosity determination plus half of the outflow time was considered as the time corresponding to the determined viscosity. The value of the initial viscosity in every experiment was determined on ficin-free blank mixtures.

The description of a typical experiment follows: Ficin (0.0800 g.) is dissolved in 2.00 ml. of distilled water by letting it stand at room temperature for 1 hour. Then 0.050 ml. of this solution is diluted to 10.0 ml. with distilled water. This solution must be used just after diluting as it decreases in activity quite rapidly because of denaturation at this concentration. One and one-half milliliters of 0.25 M cysteine (pH 7.0) are added to 0.120 ml. of this dilute ficin solution. This solution is brought to 35.0° C. (the temperature of the determination) and 13.38 ml. of 2.24% gelatin in 0.22 M buffer at 35.0° C. are added. The solution is rapidly mixed (approx. 20 sec.) and 10.0 ml. rapidly transferred to the viscosimeter. Measurements of the outflow time are started immediately. The reaction mixture of 10.0 ml. thus contains 16.0 μg. of ficin, 0.025 M cysteine, 2.00 percent gelatin and 0.20 M buffer.

TABLE 1

Rate of hydrolysis of gelatin measured by the formol titration method

Time (hrs.)	GELATIN		
	μ moles amino N liberated/ml. reaction mixture		
	pH 5.00	pH 6.72	pH 9.50
2.5	3.72	2.30	0.11
6.0	6.79	5.26	1.64
12.0	8.65	8.10	1.32
24.0	10.2	9.20	2.04
45.5	13.9	11.8	4.49
72.0	13.7	12.4	4.39

Complete hydrolysis—243 μ moles amino N.

Conditions: 20 mg. of gelatin, 1.56×10^{-3} mg. of ficin (Nutritional Biochemical) and 1.52 mg. of cysteine per ml. reaction mixture buffered with 0.05 M citrate (pH 5.0), 0.05 M phosphate (pH 6.72) or 0.05 borate (pH 9.50); incubated at 35.0° C. Titrated with 0.01 N NaOH in the presence of 9.0 per cent formalin using the Beckman Model G pH meter for determining the end point of the titration.

RESULTS AND DISCUSSION

Rate of hydrolysis of gelatin by the formol titration method. Table 1 shows the rate of hydrolysis of gelatin by ficin as measured by the formol titration method. The rate of liberation of free carboxyl groups is faster at pH 5.00 than at pH 6.72 or 9.50. This is in agreement with the results of others *(1, 12, 14, 20)*. On the other hand, Krishnamurti and Subrahmanyan *(7)* found the pH optimum for the hydrolysis of casein, edestin, egg albumin, and hemoglobin in the native and urea-denatured condition varied between 6.5 and 7.5. Winnick, Cone, and Greenberg *(19)* found the pH optimum for the hydrolysis of casein to be 7.0. This brings up the question of why the hydrolysis of gelatin should differ from the other proteins. Is is due to differences in the structure of gelatin or to the method of determining the rate of hydrolysis?

First of all, the insensitivity of the formol titration method for measurement of a small amount of hydrolysis is obvious when it is noted that 3.72 μM amino N liberated per ml. of reaction mixture corresponds to a titration increase of only 0.372 ml. of 0.01 N NaOH. For this reason, it is common practice to use an incubation time of 24 hours when measuring proteolytic activity by the formol titration method. Second, the increase in carboxyl groups (or amino groups) measures the total hydrolysis and, as usually carried out, does not measure the hydrolysis of the protein itself. This is due to the large molecular weight of the protein. In the case of gelatin, for example, 1 ml. of 2% gelatin at pH 7.0 gives a formol titration of approximately 2.00 ml. of 0.01 N NaOH. If the molecular weight of gelatin is taken as 100,000 (it actually varies from 35,000 to 100,000), each molecule must contain about 100 titratable carboxyl groups. If all the gelatin molecules were split once, the increase in formol titration would be 0.01 ml. of 0.01 N NaOH, a result much too small to detect. The molecule must be split several times before any significant incease in titration can be obtained. On the other hand, if all the molecules were split once in the middle, there would be a large decrease in the viscosity of the solution. These observations have often led to the statement that there is a purely physical change in protein during the initial stage of digestion which does not involve any chemical change. Last of all, there is evidence for the presence of exopeptidases in ficin and their activity would be measured by the formol titration method.

It is therefore obvious that if one wishes to follow the breakdown of a protein, he should use a method suitable for this purpose. The formol titration method is not suitable.

Viscosity reduction method. In the discussion of the data it is assumed that the decrease in viscosity is due to hydrolysis of the gelatin *(8)*. In preliminary work with this method it was found that the initial rate of hydrolysis was independent of the substrate concentration if the gelatin concentration was above 1.5% in the presence of 1.6 μg. ficin per ml. At 4% gelatin concentration and above, the rate decreased because of inhibition by gelatin. Therefore, a 2% gelatin solution was used throughout this work.

It was found that the relative viscosity of gelatin, up to around 5%, followed the simple logarithmic formula of Arrhenius *(2)*.

$$\log \eta_{rel} = k \times c \qquad (1)$$

where η_{rel} is the relative viscosity, k a constant, and c the protein concentration.

The logarithm of the relative viscosity of the mixture was found experimentally to be equal to the sum of the logarithms of the relative viscosities of the undigested gelatin and its breakdown products.

$$\log \eta_t = \log \eta_{gel} + \log \eta_{products} \tag{2}$$

or

$$\log \eta_t = (C_o - X)\ K_n + K_d\, X \tag{3}$$

where η_t is the relative viscosity of a partially hydrolyzed gelatin solution at time t, C_o is the initial gelatin concentration, X is the concentration of digested gelatin and K_n and K_d are the constants of Equation 1 for native and digested gelatin, respectively.

TABLE 2

Relation of relative viscosity at time t, η_t, to the relative viscosities of the undigested gelatin, η_{gel} and products $\eta_{products}$

Concentration of gelatin (per cent)	Time of outflow Seconds at 35.0° C.			log η_{gel} [1]	log $\eta_{prod.}$ [1, 3]	log $\eta_{gel+prod.}$ [1]
	Gelatin	Products	Gelatin[2] + products			
1.00	117.9	82.0	118.3	0.163	0.002	0.166
2.00	171.2	84.0	173.9	0.327	0.012	0.336
3.00	239.0	87.7	250.2	0.472	0.031	0.508
4.00	355.9	90.6	390.0	0.655	0.045	0.697
5.00	489.8	93.2	542.5	0.791	0.057	0.845
Blank on reagents		81.5				

[1] These values include the densities of the gelatin, products and reagents.
[2] Gelatin of a given concentration was completely digested by ficin and then sufficient gelatin was dissolved in the product solution to give a gelatin concentration equal to that of the original.
[3] The products contribute only slightly to the viscosity of the gelatin-ficin solution at time t. Thus log η_t is nearly equal to log η_{gel} (and thus η_t is nearly equal to η_{gel}.).

The amount of digested gelatin can be calculated from Equation 3

$$X = \frac{\log \eta_o - \log \eta_t}{K_n - K_d} \tag{4}$$

($K_n\, C_o = \log \eta_o$)

The constants K_n and K_d vary with temperature and with pH. At 35.0° C., pH 7.00 (0.2 M phosphate buffer), and 2% gelatin K_n was found to be 0.0187 and K_d was 0.0035. Mihályi *(9)* found these relations to be true also in the digestion of myosin by trypsin.

It was found that when the square of the concentration of digested gelatin, in mg., was plotted against time the data could be resolved to a simple linear relationship. Other workers *(8, 13)* have used the viscosity method, but they were unable to reduce their data to a simple linear function. Consequently, they usually expressed the enzymatic activity in rather arbitrary units such as the decrease in viscosity after 10 minutes digestion time, etc. This inability to reduce the rate of change of viscosity to a simple linear function has limited the use of this method as a simple, rapid assay method which does not require expensive apparatus.

The use of the product concentration, X, in place of the relative viscosity also has the advantage of ruling out the effect that temperature, pH, and concentration have on the initial relative viscosity. In other words, a plot of X^2 versus t will have an intercept of zero regardless of the conditions of the experiment whereas a plot of η_{rel} versus t will have various intercepts depending on the temperature, pH and concentration.

It is proposed that the reason for the linearity of the X^2 plot is due to inhibition of ficin by the products. On the basis of this assumption the following equation was derived

$$X^2 = \frac{2k_3E_0t}{K} - \frac{2}{K} \tag{4a}$$

where X is the product concentration, k_3 is the rate of splitting of the enzyme-substrate complex to form X, E_0 is the total ficin concentration, t is the time and K is the dissociation constant of the enzyme-substrate-product complex which is inactive. More work in support of this hypothesis is planned and the proposal will not be pursued further in this paper.

Effect of pH. Figure 1 shows the effect of pH on the rate of hydrolysis of gelatin by ficin. The specific reaction rate constants, K (expressed in mg.^{2} per ml.), were found to have the following values at 35.0° C. for the reaction of 16.0 μg. of ficin on 200 mg. of gelatin: pH 3.00, 1.70; pH 4.00, 2.18;

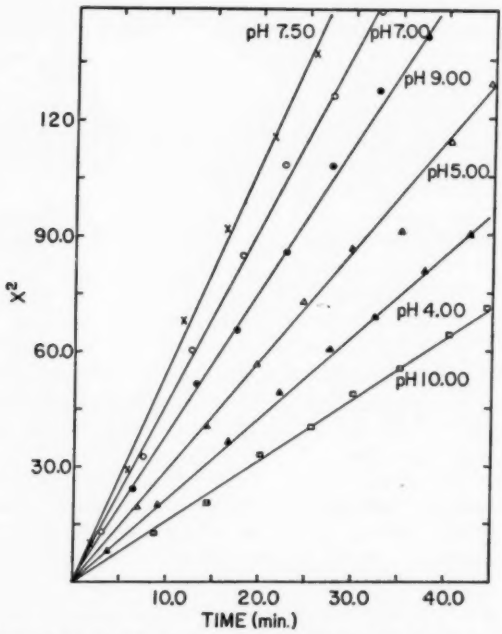

Figure 1. The effect of pH on the rate of hydrolysis of 200 mg. of gelatin by 16.0 μg. of ficin at 35.0° C. The buffers used were: pH 3–6, 0.20 M citrate, pH 7–7.5, 0.20 M phosphate, and pH 9–10, 0.20 M borate.

pH 5.00, 2.92; pH 6.00, 3.56; pH 7.00, 4.74; pH 7.50, 5.44; pH 8.00, 4.30; pH 9.00, 3.87; pH 9.50, 2.74; and pH 10.00, 1.61.

It is evident that the pH optimum is at pH 7.50 and not at pH 5.00 as indicated by the formol titration method. This brings the pH optimum for the hydrolysis of gelatin by ficin in line with those for the digestion of other proteins. Nice, linear plots are obtained from the data. However, above 60% hydrolysis, the data deviates from a straight line and becomes curvilinear. This is probably due to the reaction going from a zero order one with respect to substrate concentration to a first order reaction as the substrate is used up.

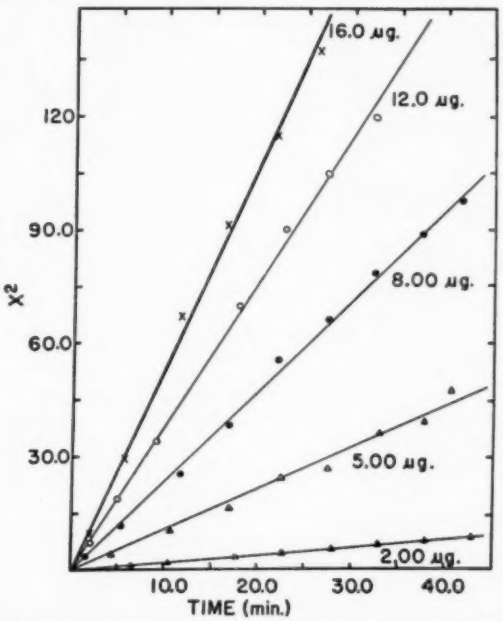

Figure 2. The effect of enzyme concentration on the rate of hydrolysis of 200 mg. of gelatin at pH 7.50 and 35.0° C.

Effect of enzyme concentration. Figure 2 shows the effect of ficin concentration on the rate of hydrolysis of 2% gelatin at 35.0° C., and pH 7.50. Again the data give linear plots. Figure 3 is a plot of the specific reaction rate constant, K (which is X^2/t), versus ficin concentration. The data do not extrapolate to zero concentration because of inhibition by gelatin. The data in Figure 4 can be reduced to the simple expression

$$C = \frac{K - b}{a} = \frac{X^2 - tb}{ta} \qquad (5)$$

where C is the concentration of ficin (in µg.), K is the specific reaction rate constant (X^2/t), X is the amount of gelatin broken down at time t, a is the slope of the curve and b is the intercept.

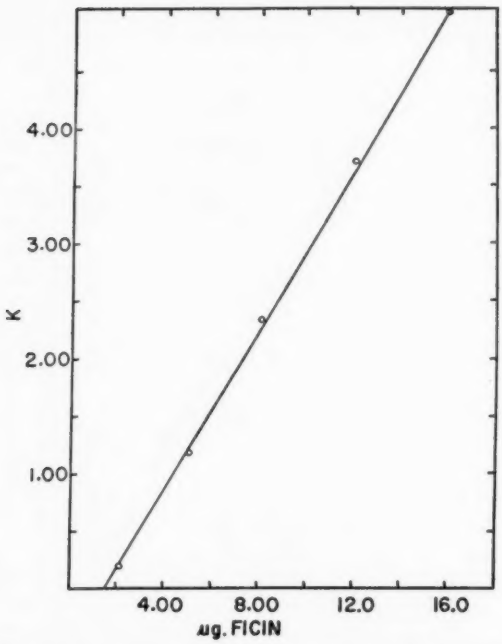

Figure 3. The effect of enzyme concentration on the specific reaction rate constant, K, obtained in the presence of 200 mg. of gelatin at pH 7.50 and 35.0° C.

Under the conditions where 200 mg. (2%) of gelatin are partially digested by ficin at pH 7.50 (0.20 M phosphate buffer) and 35.0° C. Equation 5 is

$$C\ (\mu g.) = 0.287 \frac{X^2}{t} + 1.40 \qquad (6)$$

X can readily be measured at time t, and the μg. of ficin can be calculated. For increased accuracy one can determine $X_1, X_2, \ldots X_n$ at time $t_1, t_2, \ldots t_n$. This method then offers a fast, convenient way of determining proteolytic activity.

Effect of temperature. The effect of temperature on the rate of hydrolysis of gelatin by ficin is shown in Figures 4 and 5. Again, plots of X^2 versus time give linear relationships. One might expect that the plots would be curvilinear because the amount of ficin inactivated by heat would increase with the time of exposure. However, it has been found in previous work *(18)* that there is a rapid initial decrease in activity and then further decrease in activity is very slow. This would account for the data obtained here.

Treatment of the data as in Figure 5 permits the determination of the optimum temperature and the energy of activation for the hydrolysis of gelatin by ficin. The optimum temperature was found to be 62.6° C. Above this temperature the rate of hydrolysis of gelatin fell off quite rapidly due

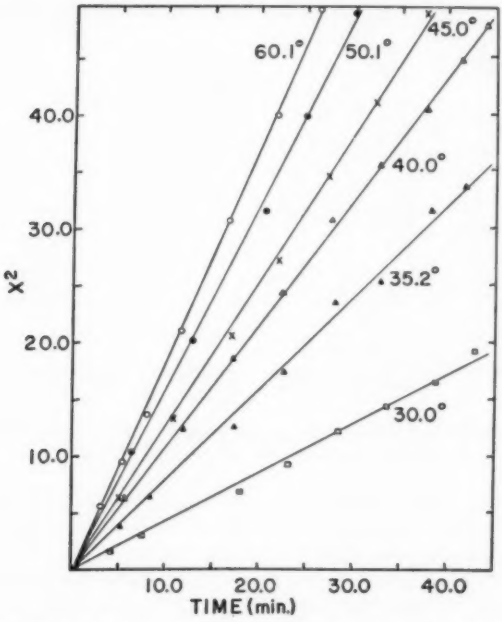

Figure 4. The effect of temperature on the rate of hydrolysis of 200 mg. of gelatin by 4.0 μg. of ficin at pH 7.50.

to denaturation of ficin. If the heat of inactivation ($\triangle H^* = -71,700$ cal. per mole) of ficin found in previous work *(18)* is used to extrapolate to a specific reaction rate constant of zero, it is found that at 80.6° C. under the conditions used here ficin has no activity. This simply means that if the ficin solution could be instantaneously brought to 80.6° C., it would be almost as rapidly inactivated. While determination of the optimum temperature is of dubious significance in the characterization of ficin, these two points are of prime importance in considerations involving ficin for the tenderization of meat. It is suggested that the conditions in the experiments here are similar to the conditions that would exist in meat and that the optimum temperature and temperature for complete inactivation of ficin would agree quite well with those actually determined on meat.

The energy of activation ($\triangle H^*$) for the hydrolysis of gelatin by ficin is found to be 6,050 cal. per mole over the temperature range of 40 to 55° C. which is in good agreement with the value of 8,000 cal. per mole for the hydrolysis of hemoglobin by papain. The energy of activation for hydrolysis of gelatin by hydrochloric acid has been reported as 25,630 cal. per mole *(3)*. Ficin has, therefore, produced a marked decrease in the energy of activation. The $\triangle H^*$ was found to be 18,000 cal. per mole over the temperature range of 30–37° C. The reason for this change in $\triangle H^*$ near 37° C. is explained by the work of Smith *(16)*. He presented data to support the theory that gelatin exists in two forms—the sol form, having a specific rotation of

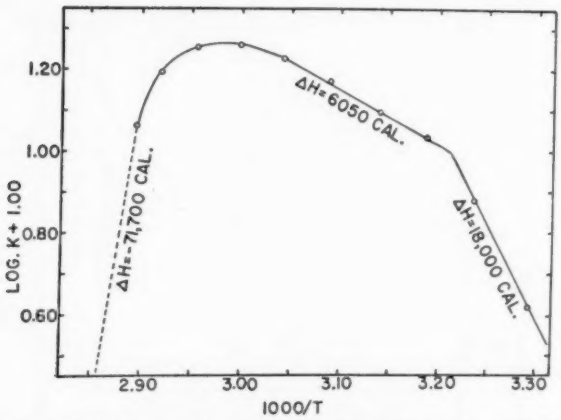

Figure 5. The effect of temperature on the specific reaction rate constant, K, obtained in the action of 4.0 μg. of ficin on 200 mg. of gelatin at pH 7.50.

−141° and being stable above 35° C., and a gel form with a specific rotation of −313° and stable below 15° C. Between these temperatures both forms are present in proportions depending upon the temperature and age of the solution. It may be assumed, therefore, that the water absorbed by the gelatin will be retained almost completely up to a temperature of 35° and the measured decrease in viscosity with increasing temperature up to this point is due to the decreasing proportion of the gel form present. Above 35° C. the decrease in viscosity produced by increase in temperature is presumably due largely to progressive loss of absorbed water *(3)*. It is suggested that $\triangle H^*$ is higher below 35° C. than it is above 35° C. because of the greater difficulty in hydrolyzing the more hydrated form of gelatin. Because of the existence of two forms of gelatin below 35° C. and the anomalous results which may be obtained, it is best to measure the effect of ficin on the viscosity at 40° C. although quite good results were obtained in this work at 35° C.

Application of the method to other proteolytic enzymes. As shown by Figure 6 the data on the hydrolysis of gelatin by papain and bromelin can be reduced to a linear form in the same manner as for ficin. Papain and bromelin were found to have the same proteolytic activity and to be only about one-half as active as the same concentration of ficin in decreasing the viscosity of gelatin. The specific reaction rate constants at 33.9° C. for papain and bromelin were found to be 2.50 and for ficin 5.10 mg.^{2} per min. The viscosity reduction method thus offers a convenient method for measuring the proteolytic activities of papain and bromelin as well as ficin.

For best results with this method as an assay method the following conditions should be observed. The gelatin solution (2.24%) buffered at pH 7.50 by 0.22 M phosphate buffer should age for at least one hour at 40° C. in a water bath maintained at ±0.1°. Determination of the activity should be carried out at 40.0° C. with a ficin solution containing the activity equivalent to 4–10 μg. of the commercial ficin used in this work. The results would be even more significant if they were expressed in terms of the activity of crystalline ficin.

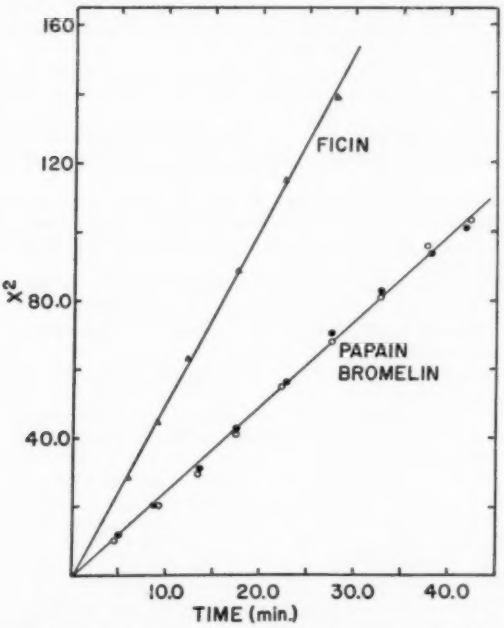

Figure 6. Comparison of the rates of hydrolysis of 200 mg. of gelatin by 16.0 μg. of ficin, papain and bromelin at pH 7.50 and 33.9° C.

SUMMARY

It has been shown that the rate of hydrolysis of gelatin by ficin can be followed by the viscosity reduction method and that the data can be made to fit a straight line by plotting the square of the product concentration versus the time of the reaction. The method offers a rapid, convenient means of measuring the proteolytic activity of ficin, papain, or bromelin.

The pH optimum for the hydrolysis of gelatin by ficin was found to be 7.5 which is similar to that reported when other proteins are used as substrates. The temperature optimum for ficin in the presence of 0.20 M, pH 7.5, phosphate buffer and a 50,000 fold ratio (g./g.) of gelatin is 62.6° C. At 80.6° C. ficin is completely inactivated. The energy of activation for the hydrolysis of gelatin by ficin was found to be 6,050 cal. per mole over the temperature range of 40 to 55° C.

Acknowledgment

The author wishes to thank Dr. D. H. Volman, Department of Chemistry, University of California, Davis, for derivation of Equation 4a.

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SPECTRAL STUDIES OF THE PIGMENTS OF COOKED CURED MEATS

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(Manuscript received April 18, 1957)

Color is one of the first quality criteria used by consumers for judging cooked cured meats. The formation of the proper cured meat hematin pigment and its stabilization is a problem of great importance to the meat preservation industry. The cured meat pigment is assumed to be a denatured globin nitric oxide hemochrome *(2, 8)*. Its color, heat stability, and oxygen lability are all characteristic of hemochromes; however, critical evaluation of this assumed composition is lacking. It is the purpose of this study to obtain better characterization of the cured meat pigment using reflectance and absorption spectra, a recently developed acetone extraction technique *(3)*, and information on the hemochromes and hemichromes of cooked meat *(1, 6)*.

EXPERIMENTAL

Cooked cured meats were purchased from local sources. Untreated slices of the cooked cured meats were used for reflectance spectrophotometry. The entire visible spectra from 380 mμ to 700 mμ were recorded using a Beckman DR recording spectrophotometer with a reflectance attachment. Acetone extracts of the heme pigments of cooked cured meats were prepared by the method of Hornsey *(3)* and their entire visible spectra from 380 mμ to 700 mμ were recorded using a Beckman DR recording spectrophotometer. The denatured globin nitric oxide hemochrome of beef was prepared in a stepwise manner by first heat denaturing ground beef, reducing with sodium hydrosulfite, and then adding 0.1% potassium nitrite. The same pigment was also prepared by first treating beef with potassium nitrite and subsequently heat denaturing the nitrited-beef.

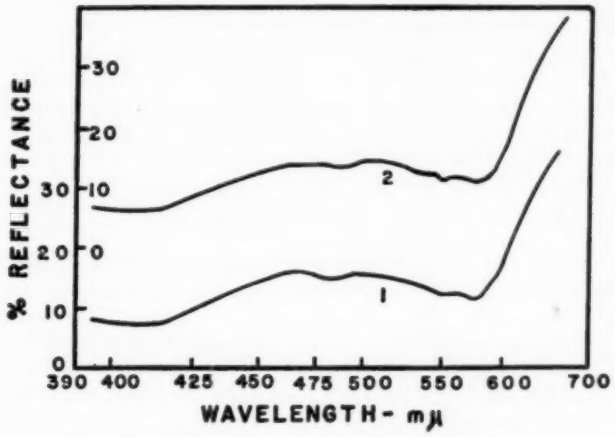

Figure 1. Reflectance spectra of cooked cured meats.
1. Smoked ham
2. Sandwich beef

RESULTS AND DISCUSSION

Good characterization of the pigments of cooked cured meats is difficult because they are oxygen labile and because their reflectance spectra are diffuse. Examples of two good spectra are given in Figure 1 and the wave lengths for the reflectance minima of the other cured meats examined are given in Table 1. Three samples appeared to be slightly oxidized in the process of recording spectra and there was no definite minimum for the beta peak but

TABLE 1

Wave-length of reflectance minima of cured meats

Cooked cured meats	Wave-length of minima, mμ			
	α	β		γ, Soret
Ham, ground No. 1	566	545(s)	480	404
Ham, smoked No. 2	580	553(s)	483	403
Ham, smoked No. 3	574	552	485	404
Ham, smoked No. 4	576	553	484	407
Ham, No. 5	574	553	482	402
Frankfurter	562	.....	483	405
Beef, sandwich	575	551	486	404
Mixed pork and beef, canned	577	552	485	400
Bacon, Canadian	572	552(s)	485	403

only a shoulder (s) in the curve. These values obtained from the position of the shoulder of the curves are less reliable than those obtained from a definite minimum. These reflectance spectra with the alpha band in the 570 mμ region, the beta band in the 550 mμ region, and the gamma band in the 400 mμ region show the characteristics of all hemochromes. The band in 480 mμ region is not a general characteristic of hemochromes *(5)* and is an unusual feature of the spectra of the cured meat pigment.

Samples of ham and bacon were oxidized with ferricyanide to produce the characteristic brown pigment of oxidized cured meats. The spectra of these oxidized pigments of ham and bacon had two bands in the visible at 550 mμ and 417 mμ and 548 mμ and 415 mμ respectively. The loss of the alpha and beta bands and their replacement with a single band at 550 mμ by oxidative conversion of the hemochromes to hemichromes is a very characteristic reaction *(5)*. Other cured meats were oxidized with hydrogen peroxide, ionizing radiation at 4×10^6 rep, and excess ascorbate. The spectra of the oxidized pigments were similar with a single band in the 540–570 mμ region and the Soret band in the 400 mμ region.

These reflectance spectral studies are direct physico-chemical evidence supporting the assumed characterization of the pigment of cured meats as a denatured globin nitric oxide hemochrome.

Spectral characteristics of the acetone extracts of a variety of commercial cooked cured meats are given in Table 2. Again, difficulty was experienced in the oxidation lability of the acetone complex of nitric oxide heme. This oxygen lability, previously discussed by Hornsey *(3)*, can be circumvented by protection from light. The results (Table 2) show that the band in the 480 mμ region is also a characteristic of the acetone complex of nitric oxide heme. The results obtained for commercial cured meat can be used for com-

TABLE 2

Wave-length of absorption maxima of acetone extracts of cured meats

Cooked cured meats extracted	Wave-length of maxima, mμ			
	α	β		γ, Soret
Ham, smoked No. 1	562	545	485	.....
Ham, smoked No. 2	569	538	480	396
Ham, canned No. 3	562	540	485	.....
Ham, canned No. 4, dark	565	538	485	.....
Ham, canned No. 4, light	570(s)	545	483	395
Frankfurter	569	540	480	396
Beef, sandwich No. 1	565	543	480	397
Beef, sandwich No. 2	569	542	483	396
Bacon, Canadian	580(s)	540	480	394
Pork ( )	563	535	476	.....
Heat denatured beef reduced with hydrosulfite and treated with nitrite	566	540	484	396
Beef treated with nitrite and then heated	566	549	485	396
Uncooked nitrited beef	565	542	484	397

parison with the hematin pigments prepared in model systems. The best evidence obtained in this research for the characterization of the cured meat pigment as denatured globin nitric oxide hemochromes came from the stepwise reaction of denatured globin hemochrome of heat denatured beef with sodium hydrosulfite to give denatured globin hemochrome and then treatment with nitrite to give denatured globin nitric oxide hemochrome. Good evidence that this stepwise reaction results in the same product as that in commercial meats and in the control system of beef treated with nitrite previous to heating are given by the spectral characteristics in Table 2.

Denatured globin hemochromes of cooked beef and cooked pork have alpha, beta, and gamma bands at approximately 560, 535, and 420 mμ, respectively, and a reflectance maxima at 495 mμ *(6)*. By comparison the denatured globin nitric oxide hemochromes examined in this study have the alpha and beta bands shifted to a longer wave length, a secondary beta peak in the 480 mμ region and a large shift of the Soret or gamma band to shorter wave length. Although the assignment of the absorbance bands in the visible region to structural configurations of the iron poryphyrins is still tentative *(7)*, it would be interesting to have an assignment of the alpha, beta, gamma band, and the band in the 480 mμ region in terms of structure of the denatured globin nitric oxide hemochrome. The Soret bands of hematin compounds may be due to the transition of pi-electrons of the porphyrin, which may be visualized as forming an "electron gas" around the porphyrin ring, to their excited level. The weaker absorption bands of the hemochromes are due to the iron and the compounds coordinated to it at the center of the porphyrin ring.

Thus, the alpha and beta bands of all hemochromes may be related to structures involving ferrous porphyrin with at least one nitrogenous compound coordinated to the iron. The 480 mμ region band of denatured globin nitric oxide hemochrome may be accounted for by the specific attachment of groups to the central iron and the valence state of this iron.

The structural configuration responsible for this absorption band probably involves nitric oxide coordinated to ferrous protoporphyrin, but the other

elements of the necessary structural configuration could not be deduced from the information available. Since this band is not present in nitric oxide hemoglobin, nitric oxide myoglobin, or simple nitric oxide hemes and hematins *(4, 5, 8)*, there must be some specific configuration around the iron and common to both nitric oxide heme acetone complex and denatured globin nitric oxide hemochrome which induces this absorption band.

SUMMARY

The cured meat pigment was characterized as a denatured globin nitric oxide hemochrome by direct reflectance spectra and stepwise formation. The spectra of the cured meat pigment have a band in the 480 mμ region—which is unusual for hemochromes.

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PROPERTIES OF THE PROTEOLYTIC ENZYMES OF COMMERCIAL FICIN

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(Manuscript received April 20, 1957)

Papain has been used for many years for the tenderization of meats, clarifying of beers, and bating of hides. Ficin has recently been used by Wang *(19)* and Miyada and Tappel *(14)* for the tenderization of meats. Wang *(19)* found that commercial preparations of ficin digested elastin over a wide range of pH values and was superior to the commercial preparations of bromelin and papain tested on a primarily qualitative basis for the purpose of tenderization of beef. He reported that crystalline ficin was only a little more active for this purpose than the crude ficin. From the standpoint of economy, any meat tenderizer containing ficin as the active principle must use crude, commercial ficin. For this reason it is important to study the properties of commercial ficin.

Although it is commonly stated that the properties of ficin from fig latex are the same as those of papain, no thorough study of the properties of ficin has been made. Robbins *(16)* reported that the active principle of fig sap is particularly effective in digesting ascaris and less effective against hookworm. He found *(17)* the pH optimum and maximum stability of the proteolytic activity to be 5.0 when gelatin was acted upon for 24 hours. These results have been confirmed by other workers using similar conditions. However, Winnick, Cone, and Greenberg *(20)*, working with crystalline ficin and using casein as substrate, found the pH optimum to be 7.0. Krishnamurti and Subrahmanyan *(8)* found the pH optimum for the hydrolysis of casein, edestin, egg albumin and hemoglobin in native and urea denatured condition varied between 6.5 and 7.5.

Walti *(18)* obtained crystalline ficin by adjusting the pH of the latex to 5.0 and allowing the solution to stand for several weeks in the refrigerator at 5.0° C. Many of the presently known properties of ficin are summarized by Greenberg and Winnick *(4)*. Indeed, it appears now that the latex of figs contains a number of proteolytic enzymes. It was reported by Nakao *(15)* that he could distinguish three proteolytic components in fig latex; a proteinase, a peptidase which acts on Witte peptone, and a peptidase specific for benzoylglycylglycine. He confirmed previous work in finding that ficin is activated by cysteine and cyanide and inhibited by monoiodo- and monochloroacetic acids, phenylhydrazine, and peptones.

Bernhard and Gutfreud *(1)* have recently worked on the kinetics of the hydrolysis of some arginine derivatives by ficin. They reported the molecular weight of ficin to be about 26,000.

EXPERIMENTAL

Ficin. The ficin used was from 2 commercial samples of crude ficin, obtained from the Mann Research Laboratories, Inc., New York, N. Y., and the Nutritional Biochemical Corporation, Cleveland, Ohio. They were found to be similar in properties and the data reported here were obtained with crude ficin from the latter source. The enzyme

solutions used in the aactivity measurements were prepared by dissolving a specified weight of the dry material in distilled water just before use. The protein concentration was checked by measuring the absorption at 280 mμ and all solutions were adjusted to the same concentration level.

Casein. The substrate was casein obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Five grams of casein were dissolved in 100 ml. of distilled water with the aid of 0.50 ml. of 5 N NaOH and heating at 90° C. for 15 minutes. This casein solution was stored in a refrigerator and was stable for at least a week. Just before use, the casein solution was adjusted to the desired concentration and pH with sufficient buffer to give a final buffer concentration of 0.2 M. The pH of the total reaction mixture was determined with the Beckman Model G pH meter and this pH value is given in all cases.

Activation and inhibition. In the activation and inhibition studies, the enzyme solutions were treated with the specified reagents for one hour at 35.0° C. *(5)*. In the other experiments where cysteine was used as the activator, it was not necessary to wait for activation as cysteine activates ficin very rapidly. The degree of activation or inhibition is expressed in each case as the ratio of the activity of the treated enzyme to that of an enzyme solution activated with 0.025 M cysteine under identical conditions.

Heat inactivation. Inactivation by heat was carried out in the following manner: test tubes (10 x 50 mm.) were equilibrated in a water bath maintained at the desired temperature. Then 0.50 ml. of the solution, containing 0.782 mg. of ficin per ml., was pipetted into each tube and the tube tightly stoppered. Samples were removed at definite intervals of time and the residual proteolytic activity determined by the method described below.

Determination of activity. The proteinase activity was measured by the Kunitz Casein Digestion Method *(9)* modified to fit the requirements of ficin. Solutions of crude ficin, usually 0.04 ml. quantities of a solution containing 0.782 mg. of ficin per ml., were pipetted into 12-ml. pyrex centrifuge tubes. To each tube was added a 0.10 ml. quantity of 0.25 M cysteine previously adjusted to pH 7.0 (or the desired amount of another activator or inhibitor). The volume of the solution in each tube was then brought to 1.0 ml. with an appropriate buffer (the final buffer concentration was 0.2 M), and the tubes placed in a water bath at 35.0° C. for 5 minutes. Then 1 ml. of casein solution, at 35.0° C., was pipetted into the first tube, and a timer started. Each subsequent tube received 1 ml. of casein solution at 30-second intervals. Three milliliters of 10% trichloroacetic acid (TCA) were added in the same order to each tube exactly 20 minutes after the addition of casein (or at definite time intervals when the rate of digestion was being measured). The tubes were removed from the bath, and the contents of the tubes were mixed thoroughly by stirring with a glass rod. They were allowed to stand for an hour at room temperature, and then centrifuged for 20 minutes. The optical densities of the supernatant solutions were read at 280 mμ using a Beckman DU spectrophotometer. The readings were corrected for the values of blanks. The blanks were prepared by first mixing 1 ml. of casein solution with 3 ml. of TCA solution, and then adding 1 ml. of the buffer solution used which contained the correct amount of ficin and cysteine. The proteolytic activity is expressed as the increase in optical density at 280 mμ produced in 5 ml. of total mixture.

RESULTS AND DISCUSSION

Effect of pH on activity. The pH activity curves for the action of crude ficin on casein are given in Figure 1. It is seen that there are two peaks of activity, one near pH 6.7 and the other near 9.5. When 1.56×10^{-2} mg. of cysteine-activated ficin were allowed to act upon 20.0 mg. of casein for 20 minutes at 35.0° C., the pH of optimum activity was near 9.5 with a smaller peak at pH 6.7. However, when 3.13×10^{-2} mg. of cysteine-activated ficin were allowed to act upon 20.0 mg. casein for 20 minutes at 35.0° C., two peaks of identical activity were obtained, one at pH 6.72 and the other at 9.50.

Figure 2 shows the rate of hydrolysis of 20.0 mg. of casein by 1.56×10^{-2} mg. of cysteine-activated ficin. Under the conditions of this experiment, the

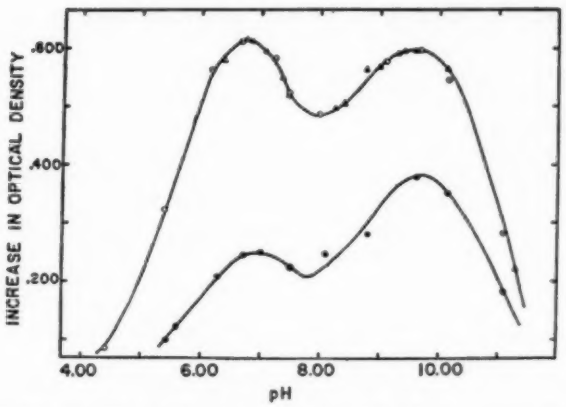

Figure 1. Digestion of casein by ficin at various pH values, concentrations of casein and at 35.0° C. for 20 minutes. The buffers were: pH 4.0-6.0, 0.2 M citrate buffers; pH 6.1-8.0, 0.2 M phosphate buffers, pH 8.1-9.8, 0.2 M borate buffers and pH 10.0 and above were 0.2 M carbonate buffers. The different amounts of substrate and enzyme are indicated as: ○—20.0 mg. casein, 3.13 x 10^{-2} mg. ficin; △—15.0 mg. casein, 3.13 x 10^{-2} mg. ficin; ▲—40.0 mg. casein, 3.13 x 10^{-2} mg. ficin; and ●—20.0 mg. casein, 1.56 x 10^{-2} mg. ficin in two ml. reaction mixtures. Activated by 0.025 M cysteine.

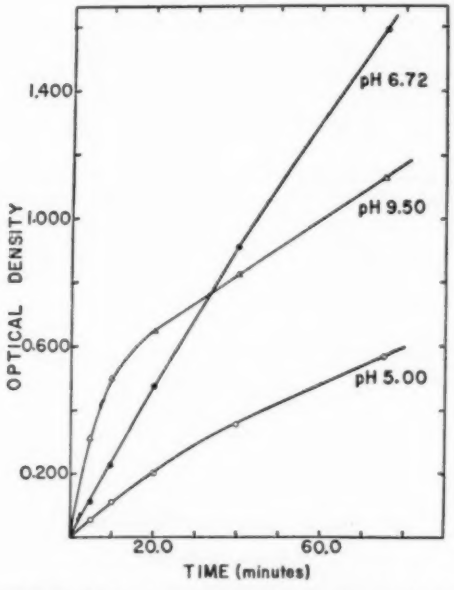

Figure 2. Rate of hydrolysis of casein by ficin. 20 mg. casein and 1.56 x 10^{-3} mg. ficin per ml. buffered with 0.05 M citrate (pH 5.0), 0.05 M phosphate (pH 6.72) or 0.05 M. borate (pH 9.50) and incubated at 35.0° C. Activated by 0.025 M cysteine.

initial rate of hydrolysis of casein is nearly three times faster at pH 9.50 than at pH 6.72 and six times faster at pH 9.50 than at pH 5.00. As the reaction proceeds the initial rate of hydrolysis of casein at pH 9.50 decreases and after 34 minutes reaction time the amount of TCA soluble material becomes less than that produced at pH 6.72 by the same reaction time. At pH 9.50 the optical density values after 2 and 4 hours incubation are 1.46 and 2.13 respectively while at pH 6.72 the values are 2.25 and 3.01 for the same intervals of incubation. (Dilutions prepared from the original sample were used for the actual measurement of optical densities in these cases.) It is clear that data based solely on incubation times in excess of 34 minutes would indicate that the optimum for digestion of casein is pH 6.72 rather than 9.50. The time at which the amount of TCA soluble material produced is the same at both pH 6.72 and 9.50 is dependent on the substrate concentration. These points have been determined to be 17 and 9 minutes for the action of 1.56×10^{-2} mg. of ficin on 10 and 5 mg. of casein per ml. respectively. The decrease in the rate of hydrolysis of casein at pH 9.50 is apparently due to limitations set by the types of peptide linkages that can be hydrolyzed by the enzyme at this pH. As noted above, the duration of the initial rate of hydrolysis is directly proportional to the initial substrate concentration. This rules out inhibition by substrate or split products as the limiting factor. Ficin, incubated under similar conditions, but in the absence of substrate, was found to be fully active after 4 hours at 35.0° C. It would appear that ficin is more specific in its action on casein at pH 9.50 than at 6.72.

This finding suggested that it would be well to study the effect of substrate concentration on the proteolytic activity. The results are shown in Figure 3. At pH 6.72 and 3.13×10^{-2} mg. of cysteine-activated ficin the maximum activity was obtained in the presence of 10–15 mg. of casein and the activity decreased at higher concentrations of substrate, presumably due to inhibition by substrate. However, at pH 9.50 and 3.13×10^{-2} mg. of cysteine-activated

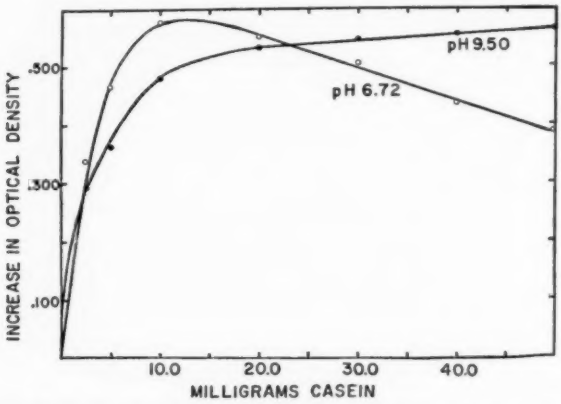

Figure 3. Effect of substrate concentration on its rate of hydrolysis by ficin at 35.0° C. Various amounts of casein were digested for 20 minutes by 3.13×10^{-3} mg. ficin (activated by 0.025 M cysteine) at pH 6.72 (0.2 M phosphate buffer) and pH 9.50 (0.2 M borate buffer).

ficin, 25–50 mg. of casein as substrate produced the maximum activity and there was no indication of inhibition of activity by substrate. However, it has been found that there is some inhibition in the presence of 100 mg. of casein at pH 9.50. At these optimum concentrations of substrate the rate of reaction was found to be directly proportional to the enzyme concentration up to 5.00×10^{-2} mg. of ficin and, at a given ficin concentration, the reaction rate was of zero order up to 30 minutes at pH 6.72 and 24 minutes at pH 9.50.

The effect of pH on the proteolytic activity was again measured using the amount of casein found necessary to produce the maximum activities in the neighborhood of pH 6.72 and 9.50. These results are also shown in Figure 1. The proteolytic activity near pH 6.72 was obtained in the presence of phosphate buffer whereas that near pH 9.50 was in the presence of borate buffer. The possibility that the buffer ions might have an influence on the proteolytic activity was tested in two ways. First, the proteolytic activity at pH 6.72 and 9.50 was determined in the presence of mixed phosphate-borate buffers and secondly, the proteolytic activity in the pH range of 7.5 to 8.5 was determined both with phosphate and with borate buffers. No essential difference in activity was obtained.

Careful examination of Figures 1, 2, and 3 will show apparent discrepancies in the optical density values for the activity of ficin at 35.0° C. and a digestion time of 20 minutes even though the same concentration of enzyme and substrate were used in all 3 cases. This should not be construed to mean that the results are not reproducible under identical conditions. The conditions varied because the ficin solutions were not allowed to stand at room temperature for the same length of time before using. Ficin solutions show the maximum activity after 1 to 4 hours standing at room temperature. Before this the activity is 3–5% lower because of the small portion which goes into solution quite slowly. After 4 to 8 hours the activity decreases because of denaturation. The period of standing was not identical in the case shown in Figure 3 with that in Figures 1 and 2.

The fall in the digestion rate, particularly on the acid side of the pH optimum, is not due to the destruction of the ficin by the prevailing acidity or alkalinity of the digestion mixtures. This was ascertained by comparing the activity of ficin after it had stood in different buffer solutions of 0.20 M for 20 minutes at 35.0° C. It was found that ficin was not inactivated in the region of pH 4 to 10. At pH 11.0 in carbonate buffer ficin lost about 10–15% of its original activity.

On the basis of the difference in behavior of ficin at different pH values it was postulated that two proteolytic enzymes are present in crude ficin. The remaining experiments were designed in part to test this hypothesis.

The enzyme-substrate intermediate. The nature of the enzyme-substrate intermediate and the dissociation constant of this intermediate can be determined by the graphic method of Lineweaver and Burk *(11)* which is based on the Michaelis-Menten theory *(13)*. A plot of the ratio of initial substrate concentration to reaction rate (S/v) against initial substrate concentration (S) yields a straight line for the case in which the enzyme-substrate complex consists of one molecule each of enzyme and substrate.

In Figure 4, S/v is plotted against S for ficin acting on casein at pH 6.72 and 9.50. It is seen that the experimental points fall fairly well along straight

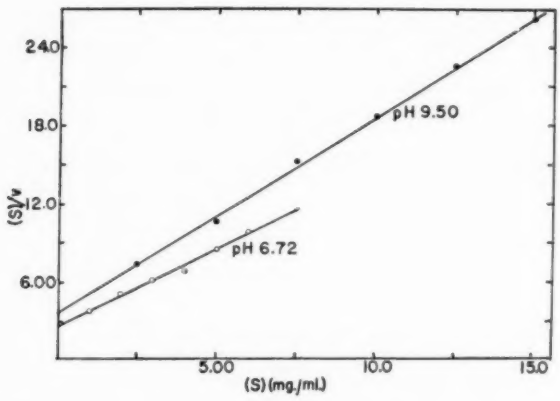

Figure 4. Determination of the Michaelis constant, K_m, of the ficin-casein complex. Various amounts of casein were digested for 20 minutes at 35.0° C. by 3.13 x 10^{-3} mg. ficin (activated by 0.025 M cysteine) at pH 6.72 (0.2 M phosphate buffer) and pH 9.50 (0.2 M borate buffer).

lines in both cases. This linear relation indicates that the intermediate complex consists of one molecule each of ficin and casein in both cases. The value of the Michaelis constant, K_m, of the enzyme-substrate complex was calculated from the data in Figure 3. K_m was found to be 2.20 mg. at pH 6.72 and 2.40 mg. at pH 9.50. When calculated in the same manner as Greenberg and Winnick *(6)* used these values are 0.22 and 0.24, respectively. Greenberg and Winnick found the K_m for the papain-hemoglobin and bromelin-hemoglobin intermediates to be 0.42 and 0.41, respectively at 30° C. and pH 7.5. One might expect the K_m values to be quite different at the two pH values *(7)*. The fact that they are very similar may have one of three possible explanations. First, two different enzymes with approximately the same K_m values at their respective pH optima may be involved. Secondly, the maximum affinity of ficin for casein may be at a point intermediate between pH 6.72 and 9.50. And last, changes in pH may not have a great influence on the K_m of ficin. These hypotheses will be tested further in this laboratory.

The effect of pH on the heat stability of ficin. Figure 5 shows the effect of pH on dilute solutions of ficin (0.782 mg. of ficin per ml.). The inactivations were carried out in the presence of 0.05 M buffer for 45 minutes at 50.0° C. in order to decrease the time involved. The effect of the buffer concentration on stability was measured and the following results were obtained in the presence of pH 7.0 phosphate buffer for 45 minutes at 50.0° C., where the first value is the molarity of the buffer, the second is the percent activity remaining in the solution: 0.00 50.0; 0.05, 58.0; 0.10, 62.5; 0.20, 68.2; 0.30, 62.0; 0.40, 62.0 and 0.50, 63.5. Ficin exhibited its maximum stability over the pH range of 5.6–7.6. Thus, for phosphate buffer concentrations up to 0.5 molar, the stability of ficin increased with increasing buffer concentration. It should also be noted that the proteolytic activities measured at pH 6.72 and 9.50 are equally affected by the pH of the medium.

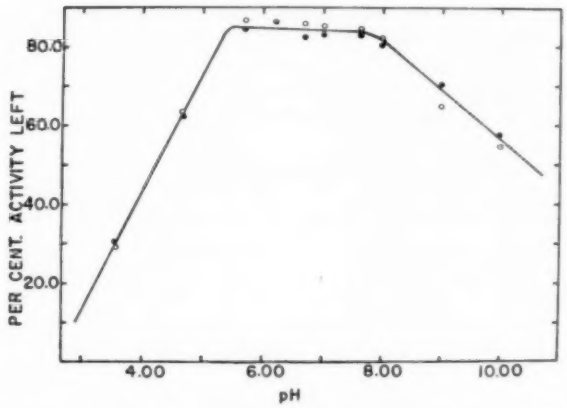

Figure 5. Effect of pH on the stability of dilute solutions of ficin. Dilute solutions of ficin containing 0.782 mg. ficin per ml. in 0.05 M buffers were held at 50.0° C. for 45 minutes, then the activity remaining was measured at pH 6.72 (○) and pH 9.50 (●) in the usual manner at 35.0° C. The buffers were: pH 3.5-6.0, 0.05 M citrate buffers; pH 6.0-8.0, 0.05 M phosphate buffers; and pH 8.1-10.0, 0.05 M borate buffers. For measurement of the activity remaining 3.13×10^{-2} mg. ficin (activated by 0.025 M cysteine) were allowed to act upon 15.0 mg. casein at pH 6.72 and 40.0 mg. casein at pH 9.50 for 20 minutes at 35.0° C.

The heat stability of ficin. The effect of temperature on the stability of dilute solutions of ficin in 0.05 M phosphate buffer at pH 7.0 is shown in Figure 6. Under the conditions of the experiment the velocity constant could be taken as proportional to the amount of active enzyme. The shape of the curves shows that the reaction is not first order when followed over a fairly long period of time, although the initial and later period considered separately may each appear to be first order. It is interesting that a linear relationship was found between the percentage of activity left (or lost) and the logarithm of the time of inactivation. Lineweaver and Schwimmer *(12)* also reported that the percentage of inactivation of papain for the first 15 minutes was unexpectedly large compared to the percentage of inactivation occurring during the next 105 minutes. They gave as one explanation of their data the possible presence of two or more proteins which have papainase activity. Greenberg and Winnick *(6)* and Winnick, Davis, and Greenberg *(21)* found that the heat inactivation of papain, bromelin, asclepain *m* and solanin followed the course of a first order reaction at certain temperatures.

There are at least two possible explanations of the data of Figure 6. It could be explained by the presence of two or more proteins which are proteolytically active and possess a difference in heat stability. There is a second explanation. Similar results have been obtained in the heat inactivation of crystalline trypsin inhibitor *(10)*, *Cypridina luciferase (2)*, and diphtheria antitoxin *(22)*. The experiments with crystalline trypsin inhibitor were unusually well planned, executed and analyzed. The data on the heat inactivation of ficin may be explained in the same manner as the data on the proteins mentioned above. Ficin, on heating, simultaneously undergoes two

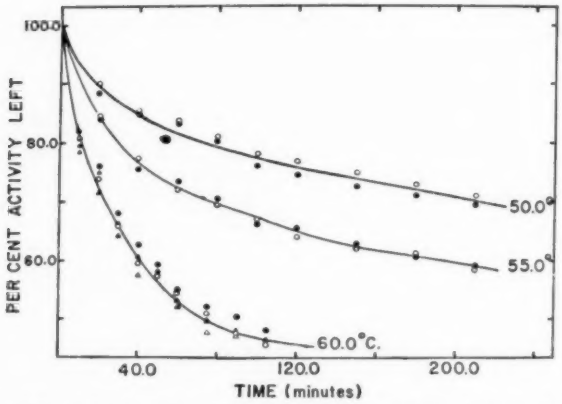

Figure 6. Effect of temperature on the stability of dilute solutions of ficin. Dilute solutions of ficin containing 0.782 mg. ficin per ml. in 0.05 M, pH 7.00, phosphate buffer were held at 50.0°, 55.0° and 60.0° C. for various periods of time. The activity remaining was then measured in the usual way at 35.0° C. The activity measured at pH 6.72 is indicated by ○ or △, at pH 9.50 by ● and ▲. The data indicated by △ and ▲ at 60.0° C. were obtained with ficin activated by cysteine before heating ○ and ● with unactivated ficin. For measurement of the activity remaining 3.13 x 10^{-3} mg. ficin (activated by 0.025 M cysteine) were allowed to act upon 15.0 mg. casein at pH 6.72 and 40.0 mg. casein at pH 9.50 for 20 minutes at 35.0° C.

reactions, one of which is reversible. If N represents the native, active ficin; I the irreversibility denatured ficin; and N_d a form into which ficin is reversibly converted, the reactions may be written thus:

$$I \xleftarrow{k_3} N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} N_d, \text{ or } I \xleftarrow{k_3} N_d \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N$$

It is difficult to distinguish between these alternative schemes on the basis of kinetic evidence.

The equilibrium ratio between denatured and native protein at a given temperature is the same regardless of whether the equilibrium is reached with ficin previously kept at lower or higher temperatures. At temperatures above a certain point, the rate of k_2 becomes negligible in comparison to k_1, and N, the amount of native protein at equilibrium, approaches zero, whereas at temperatures below a certain point the opposite is true and N approaches 100%. Under these conditions the usual first order rate equation would apply. This may explain why Greenberg and Winnick *(6)* and Winnick, Davis, and Greenberg *(21)* found that rates of heat denaturation of a number of plant proteinases, including papain and bromelin, followed first order kinetics at 60–80° C., whereas the data of Lineweaver and Schwimmer *(12)* obtained at 30° C., and the data of this paper indicate that the rates of heat denaturation of papain and ficin do not follow simple first order rate kinetics. More work on the heat inactivation of ficin is planned.

It is also seen from Figure 6 that the percentage of activity left after a given period of time was the same whether it was measured at pH 6.72 or at 9.50. It was also found that when ficin was partly inactivated by heat at pH

9.50 the percentage of activity left was the same when measured at either pH 6.72 or 9.50. This would indicate either that one is dealing with the proteolytic activity of one protein component at both pH 6.72 and 9.50 or with two proteins which are equally affected by temperature and pH. It was also found that ficin, activated with cysteine before heat treatment, was inactivated at the same rate as unactivated ficin.

The half-life periods of ficin were calculated from the data of Figure 6 by the aid of plots of percentage of activity left versus the logarithm of the time and found to be 3080 minutes at 50.0°, 526 minutes at 55.0° and 82 minutes at 60.0° C. The heat of inactivation, $\triangle H$, was calculated from a plot of the logarithm of $t_{1/2}$ versus (1/T), where $t_{1/2}$ is the half-life period of ficin at the absolute temperature T, and found to be 71,700 calories per mole. Greenberg and Winnick *(6)* found the heats of inactivation for papain and bromelin to be 75,000 and 46,000 calories per mole, respectively. While the $\triangle H$ values for ficin and papain are similar ficin does not appear to be as stable to heat as papain which was found to have a half-life of 56 minutes at 75° C. *(6)*. These results suggest that the magnitude of $\triangle H$ is not related to the degree of thermal lability of the enzyme. The $\triangle H$ of ficin resembles the values found for other plant and animal proteolytic enzymes and this indicates that the process of denaturation is similar in both plant and animal enzymes *(3)*.

Activation and inhibition. The results of the activation-inhibition measurements with ficin are given in Table 1. The behavior of ficin towards these agents, in most respects, resembles the effects that have been reported for papain and asclepain *s (21)* and for asclepain *m* and bromelin *(5)* and fig latex *(8)*. It required 0.025 M cysteine to completely activate 3.13×10^{-2} mg. of ficin in 1 ml. of 0.2 M buffer at pH 6.72 or 9.50. The amount of cysteine required for complete activation was reduced by the addition of versene. Versene, also, at a concentration of 0.025 M, increased the proteolytic activity three-fold over that of a solution of unactivated ficin. This is presumably due to the removal of inhibiting metallic ions. The maximum proteolytic activity was obtained in the presence of 0.025 M versene and 0.025 M cysteine.

A difference was found in the degree of activation of ficin by sodium cyanide at pH 6.72 and 9.50. Ficin was completely activated by 0.1 M NaCN at pH 9.50 but at pH 6.72 cyanide-activated ficin showed only 80 percent of the activity of cysteine-activated ficin even after an hour in the presence of cyanide. To determine if the difference was due to the difference in activation pH, ficin was activated by NaCN at pH 9.50 and then the pH of the solution carefully adjusted to pH 6.72 with boiled phosphate buffer to prevent oxidation of the ficin by air. Incomplete activation was still found. Thioglycolic acid, in the presence of 0.025 M versene, was found to be a better activator for ficin at pH 9.50 than an equal amount of cysteine. However, the proteolytic component at pH 6.72 was not completely activated by thioglycolic acid and versene, even when the activation was carried out at pH 9.50. On the basis of the results obtained with NaCN and thioglycolic acid there appears to be a difference in the nature of the component(s) of ficin responsible for the proteolytic activity at pH 6.72 and 9.50.

Sodium diethyldithiocarbamate and ascorbic acid did not activate ficin. They are not very strong reducing agents at these pH values and ascorbic acid is slowly destroyed in neutral and alkaline solutions.

TABLE 1
The effect of sulfhydryl activators and inhibitors on the activity of ficin

Activator	Inhibitor	Ratio of activities[1]	
		pH 6.72	pH 9.50
none	none	0.245	0.162
0.0025 M cysteine	none	0.407	.....
0.0025 M cysteine + 0.025 M Versene	none	0.842	.....
0.010 M cysteine	none	0.861	.....
0.010 M cysteine + 0.025 M Versene	none	1.00	.....
0.025 M cysteine	none	1.00	1.00
0.025 M Versene	none	0.710	0.780
0.025 M cysteine + 0.025 M Versene	none	1.05	1.08
0.025 M diethyldithiocarbamate (Na^+)	none	0.338	0.165
0.050 M diethyldithiocarbamate (Na^+)	none	0.338	0.165
0.025 M ascorbic acid	none	0.200	0.195
0.050 M ascorbic acid	none	0.192	0.194
0.025 M thioglycolic acid	none	0.475	0.570
0.050 M thioglycolic acid	none	0.495	0.569
0.025 M thioglycolic acid + 0.025 M Versene	none	0.675	1.19
0.025 M thioglycolic acid + 0.025 M Versene	none	0.785[2]	1.18[2]
0.1 M NaCN	none	0.815	1.00
0.1 M NaCN	none	0.938[2]	1.00[2]
none	0.003 M H_2O_2	0.000	0.000
0.025 M cysteine	0.003 M H_2O_2	0.370	0.328
none	0.001 M I_2	0.000	0.000
0.025 M cysteine	0.001 M I_2	0.192	0.195
none	0.02 M iodoacetic acid	0.000	0.000
0.025 M cysteine	0.02 M iodoacetic acid	0.000	0.000

The ficin solutions (3.13×10^{-2} mg.) were allowed to stand in the presence of the activator or inhibitor for 1 hour at 35° C. and pH 6.72 and 9.50 unless otherwise noted. The concentration of activator or inhibitor is for 1.0 ml. of buffered ficin solution. After inhibition by hydrogen peroxide, iodine and iodoacetic acid, the ficin was activated by 0.025 M cysteine for 15 minutes at 35° C. For measurement of activity 3.13×10^{-2} mg. ficin were allowed to act upon 15.0 mg. casein at pH 6.72 and 40.0 mg. casein at pH 9.50 for 20 minutes at 35.0° C.

[1] The activity of ficin in the presence of 0.025 M cysteine is taken as 1.00.

[2] Activated for 1 hour at pH 9.50 and 35.0° C., then the activity determined at pH 6.72 and 9.50.

The proteolytic activity of ficin was completely inhibited by hydrogen peroxide, iodine and iodoacetic acid at both pH 6.72 and 9.50. The hydrogen peroxide and iodine-inactivated ficin, but not the iodoacetic acid-inactivated ficin, could be partly reactivated by cysteine. The activation of ficin by reducing agents and inactivation by oxidizing agents and iodoacetic acid is indicative of the presence of a sulfhydryl group as an essential group for its proteolytic activity.

SUMMARY

Cysteine-activated ficin was found to possess two peaks of proteolytic activity when casein was used as substrate. These peaks occurred at pH 6.72 and 9.50 and the relative activity depended upon the substrate concentration.

The enzyme-substrate complex is believed to consist of one molecule each of ficin and casein at both pH 6.72 and 9.50. The K_m values were calculated to be 2.20 and 2.40 at pH 6.72 and 9.50 respectively.

Dilute solutions of ficin were found to possess their maximum stability in the pH range of 5.6 to 7.6 in the presence of 0.05 M buffer. The buffer concentration has an influence on the stability of solutions of ficin.

The rate of inactivation of dilute solutions of ficin by heat did not follow first order kinetics. The half-life periods of ficin at 50.0, 55.0 and 60.0° C.

were found to be 3080, 526 and 82 minutes, respectively, and the heat of inactivation was calculated to be 71,700 calories per mole.

Ficin is activated by cysteine, thioglycolic acid, sodium cyanide and versene and inactivated by hydrogen peroxide, iodine and iodoacetic acid.

Evidence is presented to support the hypothesis that two enzymes possessing proteinase activity are present in crude ficin.

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THE FERMENTATION OF GLUCOSE BY BACILLUS STEAROTHERMOPHILUS

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Bacillus stearothermophilus Donk 1920 *(7)* is one of the heat-resistant "flat sour" bacteria whose importance is almost universally recognized by the canning industry. Although confined exclusively to low acid or neutral food products, the seriousness of the spoilage caused by this organism is magnified by its sporadic, sometimes unpredictable occurrence. A further complication results because, although acids are formed, little or no gas is produced to reduce the vacuum or cause distortion of the container. Therefore, its detection by changes in acidity is impossible without opening the can, hence the common name "flat sour" spoilage. For these reasons, most of the studies on *B. stearothermophilus* have emphasized the applied aspects of its control. As a result, our knowledge of its taxonomy and physiology still is inadequate.

The taxonomy of *B. stearothermophilus* still is somewhat confused. Gordon and Smith *(14)* amended the description of this species which appeared in *Bergey's Manual of Determinative Bacteriology (3)*. However, as they pointed out, when more cultures are studied a further modification of the species description may be indicated.

Some contributions also have been made to our knowledge of the physiology and nutrition of *B. stearothermophilus*. Cleverdon, Pelczar, and Doetsch *(6)* investigated its vitamin requirements for glucose decomposition in an otherwise vitamin free medium. The amylolytic properties of various strains were studied by Campbell *(5)*, Hartman and Tetrault *(18)*, Hartman, Wellerson, and Tetrault *(19)* and Stark and Tetrault *(22, 23)*. Schmidt *(21)* observed the effects of various nutrient concentrations and salts on its ability to form spores. Fabian and Graham *(8)* tested the inhibitory effects of acetic, citric, and lactic acids, sodium chloride, glucose, fructose, and sucrose on its viability. Additional studies were undertaken by the present authors to learn more of the physiology of the organism. This report is concerned with quantitative studies on glucose fermentation by several strains of *B. stearothermophilus*.

EXPERIMENTAL

Cultures. Thirty-three cultures were used during the preliminary stages of this investigation. These included the reference culture No. 1518 obatined from Mr. C. T. Townsend, National Canners Association, Berkeley, California and 32 other strains isolated in the Department of Food Technology laboratory. The latter cultures were obtained from strained green beans (canned baby food), cream style canned corn, canned peas, commercial arrow root starch, and dry, "pablum" type baby food cereal. These cultures all had characteristics well within the limits of the amended description of *b. stearothermophilus* by Gordon and Smith *(14)*. Ten of these cultures, including No. 1518, were used for the final experiments.

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Media. The different cultures were maintained on Bacto-thermoacidurans agar or in Bacto-nutrient broth. All transfers for the fermentation experiments were one or 2 ml. portions from day-old nutrient broth cultures grown at 55° C.

The semi-synthetic medium of Cleverdon *et al. (6)* was used for the glucose fermentations. It has the following composition: casein hydrolyzate (vitamin free) 5 g.; l-cystine, 10 mg.; dl-tryptophane, 100 mg.; niacin, 1 mg.; thiamine, 1 mg.; biotin, 40 mg.; dipotassium phosphate, 5 g.; sodium chloride, 1 g.; glucose, 5 g.; and water to make 1 liter. The medium was sterilized by filtration through a Zeitz filter or autoclaved under 15 lbs. steam pressure (p.s.i.g.) for 20 minutes.

Qualitative analyses. The semisynthetic medium was used in 300 ml. portions contained in all glass fermentation flasks. The flasks were equipped with stop-cocked, capillary side arms to carry the gas to collection tubes in which the gas was trapped over saturated calcium chloride or mercury. Inoculations were made through stop-cocked thistle tubes whose delivery orifices reached almost to the bottoms of the flasks. After inoculation, the air was swept from the medium and the head-space of the fermentation flask by bubbling washed (alkaline pyrogallol) nitrogen through the medium for 15 minutes. The inoculated flasks were incubated for 4 days at 55° C. before chemical analyses were made. Two uninoculated flasks of the medium were used for control purposes in each experiment.

The free gases were analyzed in a modified Orsat apparatus. The dissolved carbon dioxide was measured in the volumetric Van Slyke apparatus. All pH values were determined with a Coleman model 18 glass electrode. The methods outlined by Neish *(20)* were used for preparation of all samples and separation of the various groups of end-products.

Paper chromatography techniques were used as aids in the qualitative determination of the organic acids. The method of Stark, Goodban, and Owens *(24)* was used for the fixed acids while the volatile acids were detected by the method of Brown and Hall *(4)*. The chromatograms of the volatile acid fractions indicated that both formic and acetic acids might be present. Formic acid first was demonstrated as a probable endproduct by mixing 1 ml. of saturated aqueous $HgCl_2$ with 1 ml. of the extracted volatile acid solution in a test tube and heating the mixture over a boiling water bath for 30 minutes. A white precipitate showed the presence of formic acid. In addition a 25 ml. aliquot of the portion containing the volatile acids was redistilled in the presence of acidified $HgSO_4$ and $MgSO_4$ to decompose the formic acid as described by Friedemann *(7)*. The distillate was titrated with 0.1*N* NaOH to the phenolphthalein endpoint. An appreciable decrease in the titratable acidity of the distillate indicated that formic acid had been decomposed. Then the neutralized distillate was concentrated on the steam bath and tested on paper *(4)*. Only one discrete spot appeared. This spot had the same Rf value (0.10) as the known acetic acid solution used as a control. A portion of known formic acid solution (0.5% aqueous) was subjected to the same redistillation treatment *(7)*. No titratable acidity remained and no spot appeared on the paper chromatogram.

Ethanol was the only compound found in the neutral volatile fraction. It was detected by the qualitative method of Feigl *(9)*. Its presence was verified by the use of two quantitative tests: the microdiffusion technique *(20)* and the method of Friedemann and Kloss *(13)*. Both quantitative methods gave reproducible results.

The qualitative study showed that the major endproducts of glucose fermentation by various cultures of *B. stearothermophilus* were lactic acid, formic acid, acetic acid, and ethanol.

Quantitative analyses. Fermentations used for the quantitative analyses were prepared as described above. The dissolved gases were determined as before. However, since no appreciable quantities of free gases were detected during the qualitative studies, this analysis was discarded. Total acidities were determined by titration of suitable aliquots of the fermented liquors with 0.1*N* NaOH to the pH values of the controls.

Reducing sugar (glucose) was determined by the modified Somogyi colorimetric method *(20)*. The percent transmittance was read on the Klett-Summerson colorimeter using a wave length of 520 mμ. The amount of glucose in the sample was determined from standard curves prepared with reagent grade anhydrous glucose.

Lactic acid was determined by the colorimetric method of Barker and Summerson *(2)* as adapted by Neish *(20)*. The isomeric activity of the lactic acid was found by

determination of the percent water of crystallization of the zinc salt as described by Hammer *(17)*. Optical rotation of the zinc lactate (4% aqueous solution) was determined in a 1 dm. tube at a wave length of 589 mμ by use of a Bausch-Lomb-Lippich saccharimeter *(25)*. Saccharimeter readings were transposed into the specific angular rotation by use of the Bates-Jackson conversion factor (0.3462).

Two methods were used for the quantitative estimation of ethanol. A microdiffusion technique using a Conway No. 1 microdiffusion unit was used for 1 ml. samples *(20)* whereas the method of Friedemann and Kloss *(13)* was used with 25 ml. samples.

The total amount of acid present in the volatile acid fraction was determined by titration. Then, because several micromethods for determination of formic and acetic acids could not be perfected, formic acid was determined by a gravimetric method *(1)* and acetic acid was calculated by difference. In experiments where the quantity of fermentation liquor was less than 100 ml. the amount of formic and acetic acids present in any sample was determined on a theoretical basis. The assumption was made that *B. stearothermophilus* carried out a mixed lactic-formic acid fermentation. Theoretically, formic fermentation produces endproducts in the following ratio: 2 formic; 1 acetic; and 1 ethanol. The gravimetric formic acid determination *(1)* substantiated this assumption.

RESULTS AND DISCUSSION

The qualitative phase of this study established that representative isolates of *B. stearothermophilus* fermented glucose under anaerobic conditions with the production of lactic, formic, and acetic acids and ethanol as the major endproducts. Carbon dioxide and the neutral volatile products diacetyl, acetoin, 2,3 butanediol and glycerol were not detected in significant amounts in any of the anaerobic fermentations, either by qualitative or quantitative means.

The quantitative phase presented a problem because the estimation of formic and acetic acids was difficult. Neither the Friedemann *(10)* nor Grant *(15)* methods for formic acid could be perfected to give reproducible results. Finally, a gravimetric method *(1)* was used with consistent success. Then acetic acid was calculated by difference from the total amount of volatile acidity. With this procedure it was possible to show that these two volatile acids were produced in the ratio of approximately 2 formic to 1 acetic. (The average for 6 duplicate determinations with 3 different cultures was $\frac{\text{formic}}{\text{acetic}} = 1.98$).

The gravimetric formic acid determination was laborious and time consuming. Furthermore, because *B. stearothermophilus* cultures cause a formic fermentation of glucose as well as a homolactic fermentation, the ratio is 2 formic; 1 acetic; and 1 ethanol. [See Friedemann *(11, 12)*; and Gunsalus and Niven *(16)*]. Therefore, after establishing this ratio most of the data for the volatile acid fractions were calculated on the theoretical basis. The determination of ethanol gave reliable results in the ratio of 1 ethanol to 2 formic when the latter was determined by the gravimetric method.

With this involved procedure quantitative carbon recoveries and calculated redox indices were obtained which substantiated the qualitative experiments as shown in Table 1. This "balance" experiment with 10 isolates including the reference strain No. 1518 was made with 50 ml. fermentations that had been incubated at 55° C. for 3 days under anaerobic conditions (N_2) in large Thunberg tubes. It is seen that lactic acid is the major end product of glucose fermentation by these isolates. Much smaller quantities of formic and acetic

TABLE 1

Products of anaerobic fermentation of glucose by strains of B. stearothermophilus

Culture Number	Controls		1518	G-3	3	5	11	12-A	1-P	6-P	9-P	2A-P
	A	B										
Products in mg. per 100 ml. of fermented medium												
Glucose present	497	497										
Glucose utilized	—	—	296	296	294	276	268	289	276	272	272	271
Lactic acid	—	—	222	227	224	190	191	212	206	202	192	204
Formic acid	—	—	26.5	27.1	26.0	25.6	24.5	26.3	25.0	24.1	24.5	25.0
Acetic acid	—	—	17.4	17.6	17.0	16.6	16.0	17.1	16.3	15.7	16.0	16.3
Ethanol	—	—	13.8	13.8	13.8	12.65	12.65	12.65	12.65	12.65	12.65	12.65
Products in mM. per 100 mM. of glucose												
Lactic acid	—	—	150	152.5	152.5	138	143	147	147.5	148.5	141.5	150.5
Formic acid	—	—	35.1	35.8	34.6	36.4	35.8	35.6	35.3	34.6	34.8	36
Acetic acid	—	—	17.6	17.9	17.3	18.2	17.9	17.7	17.5	17.3	17.4	17.9
Ethanol	—	—	18.3	18.2	18.4	18.0	18.5	17.2	17.9	18.2	18.2	18.3
Carbon recovery, %	—	—	94	96	95.5	89	91.5	93	95	93.5	90	95
Redox index	—	—	96	102	106	99	103	96	101	105	103	101
Final pH	7.2	7.2	5.0	5.0	4.8	5.1	5.4	4.9	5.2	5.3	5.3	5.4

TABLE 2

The isomer of lactic acid produced by cultures of B. stearothermophilus

Culture number	Water of crystallization of zinc lactate, %	Saccharimeter reading (zinc lactate)	Specific rotation (saccharimeter reading ÷ 0.3462)	Form of lactic acid
1518	12.35	−12.5	−3.61	L (+), dextrorotatory
G-3	12.50	−12.8	−3.70	L (+), dextrorotatory
3	12.30	−12.7	−3.67	L (+), dextrorotatory

The optical rotation of the salt was determined with a 4% solution in a 1-dm. tube at 542 mμ wave length.
Bates-Jackson factor = 0.3462.
The rotation of the salt is opposite to that of the free acid. The known $(a)_D^{20}$ = approximately 3.8 for lactic acid under similar conditions.

acids and ethanol are formed. As shown in Table 2, the lactic acid is the dextrorotatory (L+) isomer.

Fermentations conducted with 2 liters of the semisynthetic glucose medium under anaerobic conditions showed that the conversion of glucose by representative isolates of *B. stearothermophilus* was very rapid at 55° C. As seen in Figure 1 the fermentation by strain No. 1518 is nearly complete 24 hours after inoculation with 2 ml. of 24 hour culture. These data are typical for all of the cultures studied and emphasize the very rapid growth characteristics of the species. They also stress the small amount of volatile acids produced in comparison with the lactic acid formed by the various strains of *B. stearothermophilus* when grown in a neutral to acid medium.

Gunsalus and Niven *(16)* found that members of the enterococci formed large quantities of formic and acetic acids and ethanol in the ratio 2:1:1, respectively, during fermentation of glucose in an alkaline, buffered medium. The possibility existed that the formic fermentation of glucose by *B. stearothermophilus* would be affected by the pH of the medium in the same manner as that of the enterococci. Fermentations were conducted in 50 ml. quantities

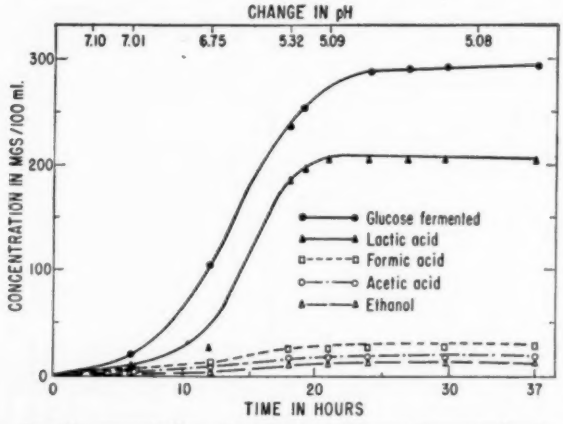

Figure 1. Graphic presentation of products of glucose fermentation by strain 1518, B. stearothermophilus.

of the glucose medium incubated under anaerobic conditions (N_2) in Thunberg tubes at 55° C. for 3 days. Three cultures including strain No. 1518 were tested in the medium buffered at pH 5.8 and 7.8, respectively, with Sørensen's 1/30 Molar KH_2PO_4–Na_2HPO_4 buffer solutions. The buffer solutions replaced the distilled water in preparation of the two media. The results in Table 3 show the marked effect of pH on alterations in the yield of formic and acetic acids and ethanol by the 3 cultures of *B. stearothermophilus*. The fermentation of glucose was practically complete in the medium adjusted to 7.8 and the production of formic and acetic acids and ethanol was doubled.

CONCLUSIONS

A study of the anaerobic conversion of glucose by various strains of *B. stearothermophilus* grown in a semisynthetic medium at 55° C. indicates that the species causes a homolactic fermentation with dextrorotatory (L+) lactic acid as the main end product. In addition a formic acid fermentation occurs in which the three volatile end products are produced in the ratio of 2 formic acid to 1 acetic acid and 1 ethanol but in much smaller quantities than the lactic acid.

TABLE 3

The effect of pH on the anaerobic fermentation of glucose by strains of B. stearothermophilus

pH of medium	5.8				7.8			
Culture number	Control	1518	G-3	3	Control	1518	G-3	3
Products in mg. per 100 ml. of fermented medium								
Glucose present	497	—	—	—	496	—	—	—
Glucose utilized	—	50	48	45	—	461	471	473
Lactic acid	—	38	38	46	—	288	291	291
Formic acid	—	3.45	3.45	3.18	—	71.1	77.0	77.7
Acetic acid	—	2.22	2.22	2.10	—	46.3	50.2	50.6
Ethanol	—	1.73	1.73	1.73	—	36.8	36.8	34.6
Products in mM. per 100 mM. of glucose								
Lactic acid	—	152	158	160	—	125	124	123
Formic acid	—	27	28.1	28	—	60.5	62.5	64.4
Acetic acid	—	13.3	13.9	14	—	30.2	32.0	32.2
Ethanol	—	13.5	14.1	15	—	31.3	30.6	28.7
Carbon recovery, %	—	90	94.7	95.5	—	96	97	95.5
Redox index	—	1.00	0.996	0.935	—	1.04	1.02	1.12
Final pH	5.8	4.8	4.8	4.8	7.8	6.2	6.1	6.1

The decomposition of glucose by cultures of *B. stearothermophilus* is markedly increased under alkaline conditions. Under these conditions the formic fermentation products increase whereas lactic acid production decreases.

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DETERMINATION OF SOLUBLE WHEY PROTEIN BY DIRECT NESSLERIZATION OF KJELDAHL DIGESTS[a]

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Heating fluid milk may denature all or part of the whey proteins and render them precipitable with the caseins by rennet, acid, or saturated salt *(13, 14)*. Whey protein denaturation in fluid milk is dependent upon time and temperature of heating, and it has been suggested that therein lies an index to heat treatment *(2, 4, 6)*. The amount of soluble protein nitrogen in the whey after precipitation of the caseins and denatured whey proteins may be used as the heat treatment index. Percent denaturation is a more exact index, but requires a knowledge of both total soluble whey protein nitrogen in the unheated milk and total soluble whey protein nitrogen in the heated milk *(11)*.

Although several methods have been published for determination of whey proteins *(3, 7, 10, 13, 15, 16)*, the turbidimetric method of Harland and Ashworth is widely used as a control procedure in the manufacture of dry milk *(11)*. This is attributable to its relative simplicity, as compared to the other methods, which require considerable time or specialized equipment. Collaborative tests by the Harland and Ashworth method, however, showed that differentiation of nonfat powders for use in breadmaking was fairly reliable, but poor agreement existed on absolute levels of soluble whey protein reported *(1)*.

It was thought that determination of soluble whey protein nitrogen in the solids-not-fat of milk and milk products by direct nesslerization would be quantitative and relatively easy to perform. In the method which was developed the soluble whey proteins are precipitated by zinc and NaOH *(8)*, digested by H_2SO_4, and nesslerized in the same tube. Precipitation is by a slight modification of the rapid procedure which was used by Somogyi *(17)*, for deproteinization of blood.[c] This paper is confined to a description of the method, and its application for determining total soluble whey protein in milk and dry milk products.

MATERIALS

Equipment:

Equipment for making fat and total solids determinations in milk and milk products (Mojonnier testing equipment was used).

Constant temperature bath set at 40° ± 1° C.

Centrifuge for operation at 1200 to 1500 r.p.m. (International size 1 type S-B with an 8 place head was used. To support the top ends of the 25 x 200 mm. tubes, two No. 10

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[a] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Mo., June 10–14, 1956.

[b] Contribution from the Department of Dairy Science.

[c] Somogyi *(18)* reported that zinc filtrates prepared by this method could be used for determination of non-protein nitrogen in blood. Uric acid, however, was entirely absent in the filtrates.

stoppers were fitted to the spindle which holds the head. This arrangement permitted centrifuging of 4 tubes at a time).

Spectrophotometer. (Coleman Universal Model 14 with 19 x 105 mm. round cuvettes was used).

Pyrex test tubes, 25 x 200 mm. (Folin-Wu blood digestion tubes marked at 35 and 50 ml. are recommended.)

Sand bath, 7 inches dia. and 1 inch deep containing 0.5 inch of sand.

Ring, 7 inches dia. and ringstand. The ring was covered with 20 mesh screen in which 6 openings of 25 mm. dia. were made for supporting the digestion tubes.

Vacuum line with about 12 inches of vacuum.

Volumetric pipettes, sizes 1, 2, and 5 ml.

Reagents:

Sodium chloride, granular, reagent grade.

Zinc sulfate solution ca. 5%; prepared by dissolving 5.0 g. of zinc sulfate in 100 ml. of distilled water.

Sodium Hydroxide solution, ca. $\frac{N}{2}$ normal.

Sulfuric acid, C. P. concentrated, S. G. 1.84.

1:1 Sulfuric acid solution prepared by adding 1 volume of concentrated, C. P., sulfuric acid to 1 volume of distilled water.

Standard ammonium sulfate solution, a solution containing 0.10 mg. nitrogen per ml. was used.

Hydrogen peroxide 30%, certified reagent.

Nessler's solution, Koch and McMeekin modification *(5)*. (Available from Hartman-Leddon Co., Philadelphia, Pa.).

Ammonia-free distilled water.

METHODS

The procedure is presented schematically in Figure 1. Percent total milk solids and percent fat are determined *(12)*, and percent solids-not-fat is the difference between these values. Then, in a 300 ml. Erlenmeyer flask, a representative sample of the product is standardized with distilled water to contain 8.0 g. of solids-not-fat and 92 g. of water.

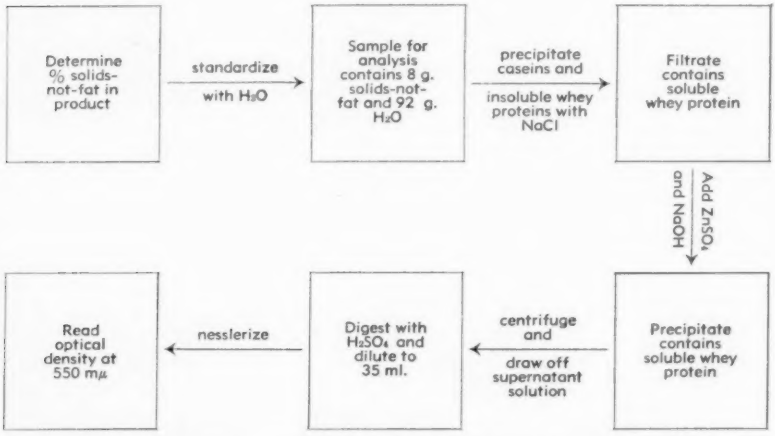
Determine % solids-not-fat in product
standardize with H_2O
Sample for analysis contains 8 g. solids-not-fat and 92 g. H_2O
precipitate caseins and insoluble whey proteins with NaCl
Filtrate contains soluble whey protein
Add $ZnSO_4$ and NaOH
Precipitate contains soluble whey protein
centrifuge and draw off supernatant solution
Digest with H_2SO_4 and dilute to 35 ml.
nesslerize
Read optical density at 550 mμ

Figure 1. Diagram of Direct Nesslerization Method for Soluble Whey Proteins.

The caseins and insoluble whey proteins are precipitated by a slight modification of the procedures of Harland and Ashworth *(3)*. Mix and warm the standardized sample to 40° C. Add 40 g. of NaCl; shake vigorously in a vertical motion for 40 seconds; immerse for 30 minutes without further agitation in a 40° C. water bath; and filter through No. 2 Whatman paper. While the whey is filtering, the funnel is supported on the rim of a 100 ml. cylinder which is provided with a ground glass stopper. The top of the funnel is kept covered with a watch glass, and the cylinder is stoppered immediately after filtration.

Transfer an aliquot volume of filtered whey into a 25 x 200 mm. pyrex test tube. One ml. of whey is used if the whey was separated from unheated milk, from "low heat" [4] nonfat dry milk, or from "medium heat" nonfat dry milk. Three ml. of whey are used if the whey was separated from "high heat" nonfat dry milk.

A reagent-blank test and 2 blanks for standard tests are started at this time by substituting H_2O for the whey in each test. They are run by the procedure for running the tests containing whey, except that after digestion each blank for the standard tests is dissolved in 10 ml. of H_2O and designated amount of standard $(NH_4)_2SO_4$ solution is added.

Dilute each test to 6 ml. with H_2O. Add 1 ml. of $ZnSO_4$ solution, and while swirling, 0.6 ml. of $\frac{N}{2}$ NaOH. The mixture must be alkaline to precipitate the proteins at room temperature. The exact amount of the NaOH solution required is predetermined by titrating to the phenolphthalein endpoint 1 ml. of the $ZnSO_4$ solution plus 6 ml. of sample mixture of whey and water.

Allow each precipitate to stand undisturbed for 5 minutes. Rinse the inside of the tube with about 5 ml. of H_2O from a wash bottle. Centrifuge at 1200 to 1500 r.p.m. for five minutes. The precipitate must form a gelatinous mass which adheres well to the bottom of the digestion tube.

Draw off the solution immediately through capillary tubing with about 12 inches of vacuum. Tilt the tube slightly and do not permit any of the precipitate to be removed with the solution.

Add 5 ml. of H_2O to the precipitate; mix by swirling; and rinse the sides of the tube with 2 to 3 ml. of H_2O from a wash bottle. Centrifuge and draw off the solution as above.

Add 0.5 ml. of concentrated H_2SO_4 and a glass bead to each mixture. Place the tubes on the sand bath through the holes in the screen at an angle of about 45°, Figure 2. The depth of the sand in the bath is about 0.5 inch.

Heat slowly with occasional jarring of the tubes by tapping with a spatula until the water is evaporated. Continue digestion for 10 minutes after the digestion mixture is charred and white fumes of H_2SO_4 fill the tube. Then remove from the sand bath; allow to cool for about one minute; and add 1 to 2 drops of 30% H_2O_2 directly into the mixture. Replace on the sand bath, and heat for ten minutes, being certain that all of the H_2O_2 is expelled and the mixture is water clear.

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[4] "Low heat", "medium heat", and "high heat" are terms used to designate different types of nonfat dry milk and generally indicate the relative preheat treatment given the fluid milk. These products, however, have not been clearly defined.

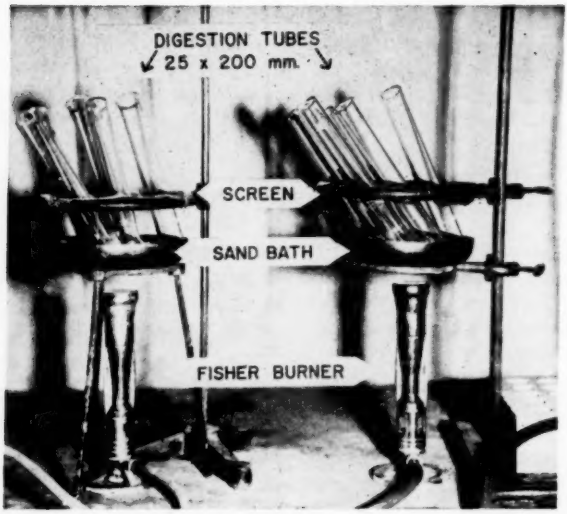

Figure 2. Sand bath for heating digestion mixtures for determination of soluble whey protein nitrogen.

Allow to cool to room temperature. Finish preparing the standard tests by adding ten ml. of H_2O to each of the two blanks which were digested for the standards. Then add 4 ml. of standard $(NH_4)_2SO_4$ solution to one tube and 6 ml. to the other tube, thus making two standard tests containing .4 mg. and .6 mg. of ammonia nitrogen.

Dilute each mixture to 35 ml. with ammonia free H_2O and nesslerize by pouring 15 ml. of Nessler's solution directly into the tube. Immediately invert four times and allow to stand 6 to 8 minutes for maximum color development.

The pH of the final Nessler color should be in the range of 13.5 to 14.0, and tests which have any indication of turbidity should be discarded. If turbidity should occur, the addition of 2 to 3 drops of 1 : 1 H_2SO_4 to the diluted digest just before nesslerizing may correct the pH of the mixture and eliminate turbidity development.

The spectrophotometer is zeroed against distilled H_2O *(9)* and optical density of the Nessler color is read at 550 mμ wave length. The nitrogen concentration is determined by referring to the standard graph, Figure 3. The reagent-blank test should not show an optical density greater than .035 and the standard tests should recover 100 $\pm$ 5 percent of the nitrogen added. Also, the standard tests should not vary more than $\pm$ 0.1 mg. of nitrogen from that found in the sample analysis. This requirement will generally be met when the suggested amounts of whey are used for analysis and when the standards contain .4 and .6 mg. of nitrogen.

The optical density of the Nessler color in the plot, Figure 3, is obtained by digesting a series of blank runs, dissolving each digest with 10 ml. of H_2O, adding known amounts of standard $(NH_4)_2SO_4$ solution, diluting to 35 ml., nesslerizing, and determining optical density as in the above procedure.

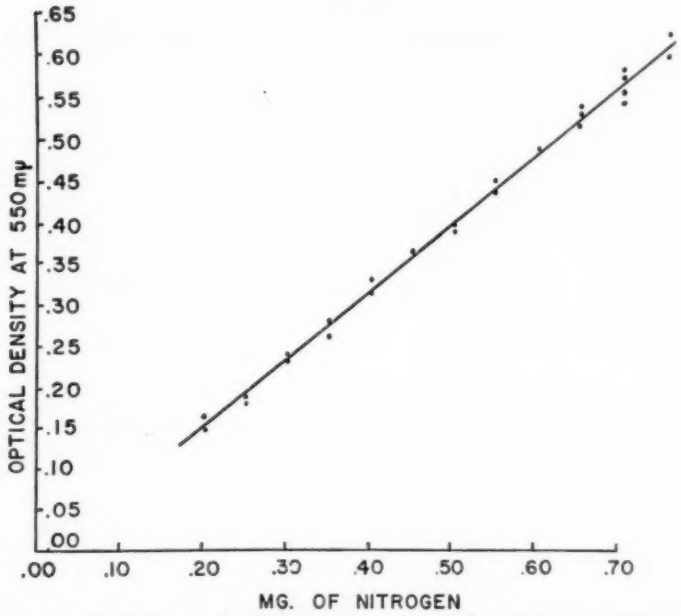

Figure 3. Standard graph for determining nitrogen by direct nesslerization.

The mg. of nitrogen found in the sample is divided by the number of ml. of whey used in the test to give the mg. of nitrogen per ml. of whey. Then calculate the mg. of nitrogen per gram of solids-not-fat by multiplying the mg. of nitrogen per ml. of whey by the factor 13.59.

The factor 13.59 is the ml. of whey per gram of solids-not-fat when the whey is separated by the above procedure. It is ⅛ of the total whey volume, and total whey volume is the volume of whey retained in the precipitate and paper plus the volume of whey collected in the cylinder.

The volume of whey in the precipitate and paper was calculated from the density and percent moisture in the filtered whey and from the g. of H_2O in the precipitate and paper, viz.

1. Wt. (g.) of retained whey $= \dfrac{\text{g. } H_2O \text{ in ppt. and paper} \times 100}{\%\ H_2O \text{ in Whey}}$

2. Vol. retained whey $= \dfrac{\text{wt. (g.) of retained whey}}{\text{density of filtered whey}}$

3. Factor $= \dfrac{\text{vol. retained whey} + \text{vol. measured whey}}{8}$

RESULTS AND DISCUSSION

Data for six factor determinations are shown in Table 1.

The standard error of estimate for analysis of whey from different milk products for soluble whey protein nitrogen is shown in Table 2. The estimated

TABLE 1

Factor determination from measured and retained whey

Sample	Measured whey			H_2O in PPT. and paper	Retained (calculated) whey		Retained plus measured whey	Factor
		Density	Moisture					
	ml.	*gm/ml.*	%	*gm.*	*gm.*	*ml.*	*ml.*	*ml.*
R1[1]	75.0	1.2140	70.3	28.7	40.8	33.6	108.6	13.58
P1[2]	78.0	1.2136	70.3	26.1	37.1	30.6	108.6	13.58
P2	75.0	1.2143	70.3	28.9	41.1	33.8	108.8	13.60
P3	66.6	1.2165	70.3	36.0	51.2	42.1	108.7	13.59
P4	82.0	1.2155	70.4	22.9	32.5	26.8	108.8	13.60
P5	31.4	1.2146	70.3	23.3	33.1	27.3	108.7	13.59
Average								13.59

[1] R designates raw milk.
[2] P designates dry milk.

error in mg. of nitrogen per g. of milk solids-not-fat was numerically higher when unheated milk or "low heat" nonfat dry milk was analyzed than when "medium heat" or "high heat" nonfat dry milk was analyzed. As shown by the coefficient of variability, however, the relation of the estimated error of a single test to the mean of the data used for calculating the respective error was lower for unheated or "low heat" nonfat dry milk than for "medium heat" or "high heat" nonfat dry milk. This demonstrates that the relative error of estimate is less for products containing higher amounts of soluble whey protein nitrogen than for products containing lower amounts of soluble whey protein nitrogen. The precision, however, is good for all products tested.

This method permits rapid, quantitative determination of total soluble whey protein in dairy products which can be standardized to contain 8.0 g. of solids-not-fat and 92 g. of H_2O. Several samples may be analyzed together, thus decreasing the test time per sample. The data are expressed as part of the solids-not-fat which is made constant in each analysis by standardization. This permits a direct comparison of data from milk products of different composition. Results of analysis of eight different products appear in Table 3.

The total soluble whey protein in the solids-not-fat of the milk products analyzed varied between 56.1 and 5.0 mg. inclusive. Since information on the

TABLE 2

Standard error of estimate for protein nitrogen in whey from milk products of different preheat treatments

Product type	Number of tests	Whey sample size	Arithmetic mean	Standard error for:		Coefficient of variability[4]
				Single test	Ave. of duplicates	
	Duplicates	*ml.*	*mg/g/s.n.f.*[3]	*mg*	*mg.*	%
Low heat[1]	24	1	8.42	±.207	±.147	2.46
Medium heat[2]	8	1	4.88	±.124	±.087	2.53
High heat[2]	13	3	1.59	±.067	±.047	4.21

[1] Unheated milk and nonfat dry milk products.
[2] Nonfat dry milk products.
[3] s.n.f. = solids-not-fat.
[4] $\frac{\text{standard error for single test}}{\text{arithmetic mean}} \times 100$.

TABLE 3

Soluble whey protein nitrogen and protein in unheated milk and dry milk products

Product type	Soluble whey protein nitrogen			Soluble whey protein			
	mg/ml of whey[3]	mg/g. s.n.f.[4]	S. E.[3]	mg/g. s.n.f.[5]	S. E.[3]	Percent in s.n.f.	g/100 g. milk[6]
Unheated composite whole milk[1]	.64	8.7	±.147	55.51	±.938	5.55	.444
Unheated composite skimmilk[1]	.65	8.8	±.147	56.14	±.938	5.61	.449
Low heat NDM[2]	.62	8.4	±.147	53.59	±.938	5.36	.429
Low heat NDM[2]	.51	6.8	±.147	43.38	±.938	4.34	.347
Medium heat NDM[2]	.29	3.9	±.087	25.45	±.555	2.55	.204
High heat NDM[2]	.13	1.7	±.047	11.04	±.300	1.10	.088
High heat NDM[2]	.08	1.1	±.047	7.02	±.300	0.70	.056
Dry whole milk[2]	.11	1.6	±.047	10.08	±.300	1.00	.080

[1] VPI dairy herd.
[2] Commercial products.
[3] Ave. of duplicate tests.
[4] s.n.f. = solids-not-fat.
[5] Nitrogen x 6.38.
[6] Containing 8.0 g. s.n.f. and 92 g. water.

preheat treatment of the fluid milk during manufacture of the commercial dry milk products was not available, the exact relation of preheat treatment to total soluble whey protein content is not shown by these data. The data demonstrate, however, the wide range over which total soluble whey protein may vary in milk products of different preheat treatments. They also point out that the extent of decrease in total soluble whey protein may reflect preheat treatment of fluid milk.

SUMMARY

A direct nesslerization method for the determination of total soluble whey protein in milk and milk products is described. The soluble whey proteins are extracted in saturated salt solution, and are precipitated with zinc, digested with H_2SO_4, and nesslerized in the same tube. They are expressed as part of the milk solids-not-fat, making the data from different products comparable. The amount of total soluble whey protein in milk products which were made by different preheat treatments varied over a wide range. It was highest in unheated fluid milk and lowest in "high heat" nonfat dry milk.

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COMPLEMENTARY EFFECTS OF HEAT AND IONIZING RADIATION [a, b, c]

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It has been suggested by Brasch, Huber and Waly *(3)* and by Morgan and Reed *(9)*, that a combination of heat and ionizing radiations might be used to process food. Such a combination might reduce off-flavor production due to radiation doses and serve, additionally, in the inactivation of radio-resistant enzymes. A combined heat and radiation treatment would therefore solve, if effective, two of the most serious problems of food irradiation and eliminate the need to resort to a full heat process.

Although this technique has been considered mainly from the point of view of its contribution to radiation preservation, it is also possible to regard it as an adjunct to conventional heat processing. For large products, such as canned ham, which must be heated by conduction, relatively small doses of radiation could perhaps be used to reduce the total heat process normally required. Thermal penetration would, however, remain a problem.

A quantitative study of the effects of combined heat and radiation on spores of thermophilic anaerobe No. 3814, putrefactive anaerobe No. 3679 and the acid tolerant flat sour No. 2253 was made by Morgan and Reed *(9)*. They used gamma ray doses of 250 K rep, 600 K rep and one million rep and temperatures of 212° and 240° F. They found a synergism only when irradiation preceded heating. Another quantitative study of this effect was made by Kempe *(8)* using spores of *Clostridium botulinum* strains 62A and 213B and the unidirectional nature of the effect was confirmed.

METHODS AND MATERIALS

P. A. No. 3679 spore propagation. *Cameron's Putrefactive Anaerobe No. 3679*, a strain of *Clostridium sporogenes* (from a culture originally obtained from the National Canners' Association) was cultured in the following manner:

One pound of beef liver was ground in a meat grinder, mixed with one liter of distilled water and simmered for an hour. The boiled material was filtered through cheesecloth and the cake was saved. The liver infusoin was made up to one liter. To this infusion was added 10 g. of peptone and 1 g. of K_2HPO_4 and the pH of the medium was adjusted to 7.4. The medium was transferred to test tubes and flasks containing clean iron nails and then was sterilized.

The spores were first grown in this medium in 40 x 250 mm. test tubes under vaseline stratification. These cultures were transferred, after 2 days' incubation at 37° C., to 250 ml. Erlenmeyer flasks and incubated at 30° C. for 2 days. Finally, all cultures were transferred to a liter flask and incubated for 2 weeks at 30° C.

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[a] Contribution No. 310 from the Department of Food Technology, M. I. T.

[b] Presented at the Seventeenth Annual Meeting of the IFT, Pittsburgh, Pa., May 14, 1957.

[c] Part of a thesis by B. Kan submitted in partial fulfillment of the requirements for the degree of Ph.D., Department of Food Technology, M. I. T.

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Bacillus cereus var. terminalis propagation. *Bacillus cereus* var. *terminalis* spores were grown by shake flask culture using medium "G" of Stewart and Halvorsön *(12)* and a culture obtained from Professor H. O. Halvorsön. After 2 days' incubation at 30° C. the spores were harvested and washed three times centrifugally, suspended in physiological saline, and stored in a refrigerator. These spores were "heat shocked" by heating for 10 minutes at 70° C. in a water bath, just prior to irradiation.

Irradiation procedures. In the majority of experiments reported here the bacterial suspensions were irradiated by gamma rays from a Co^{60} source *(7)*. The "heat shocked" bacterial suspensions were irradiated in sealed Pyrex glass ampoules before being transferred to the capillary melting point tubes for heat treatment. In the experiment to determine the effects of heating on spore radiosensitivity the unheated spore suspensions were poured into capillary tubes, heated in the Stern apparatus (vide infra), and irradiated in bundles in the Co^{60} source. In the final experiment with ham purée in cans the inoculated purée, packed in topless 211 x 011 drawn cans sealed in polyethylene sleeves, was irradiated with cathode rays from a Van de Graaff accelerator.

Heating equipment. The heat equipment used for these studies is described by by Stern *(10)* and Stern and Proctor *(11)*. The equipment consists of 2 stainless steel pans with a solenoid actuated device that enables the transfer of a sample holder from a position over the hot mineral oil bath to a position over a cold water bath in 0.4 seconds. The capillary tube samples used in these experiments were inserted through holes in the sample holder and protruded into the heating or cooling medium so that the bacterial suspensions in the tubes were entirely immersed. Experiments by Stern and by Farkas *(6)* have determined the heating lag in these soda glass (1.5–2.0 x 100 mm.) tubes to be 6 seconds before a point 0.1° C. below the bath temperature is reached.

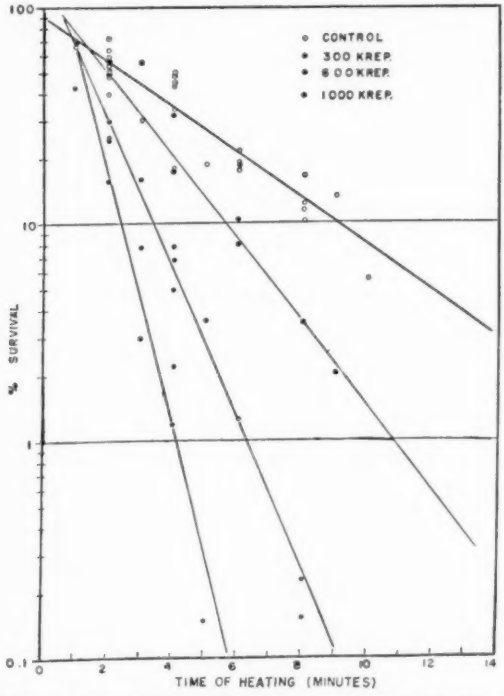

Figure 1. Effect of time of heating at 110° C. on P. A. No. 3679 spores that have been previously treated by 3×10^5, 6×10^5, and 1×10^6 rep of gamma radiation from a Cobalt-60 source.

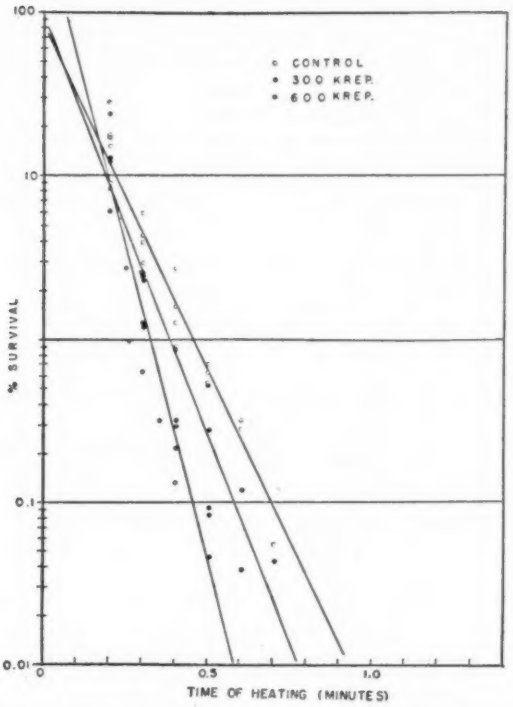

Figure 2. Effect of time of heating at 120° C. on P.A.No. 3679 spores that have been previously treated by 3×10^5 and 6×10^5 rep of gamma radiation from a Cobalt-60 source.

Preparation of capillary tube samples. Soda glass melting point tubes were soaked in chromic acid cleaning solution overnight, were rinsed carefully in distilled water and were dried. Using a gas-oxygen flame the center portions of the tubes were fused to form a glass bridge. One end of each tube was bent at right angles to the body in order to position the tube in the sample holder. The tubes were sterilized overnight in a hot air oven at 120° C. Three one-hundredths ml. of appropriately treated bacterial suspensions was placed in the middle of the lower section of each tube using a 1 ml. tuberculin syringe fitted with a 24-gauge, 1½ inch hypodermic needle, and the open end was sealed by drawing off the tip in a microburner.

Counting of capillary tube samples. Capillary tube samples for counting were first washed in acetone to remove any adherent oil. They were then soaked for one minute in chromic acid cleaning solution, rinsed under the cold tap, and finally, rinsed in sterilized distilled water. The portion of the tube below the glass bridge, after being cleaned as above, was broken off into a dilution bottle or tube containing either 99 ml. or 9 ml. of sterile, distilled water. A sterile glass rod was used to crush the sample in the sterile water, thereby releasing the bacterial suspension for counting.

Viable counts were made using Andersen's medium *(1)* for the *P.A. No. 3679* spores and Difco Nutrient Agar for the *B. cereus*. Incubation was at 37° C. for 48 hours for *P.A. No. 3679* and for 24 hours for *B. cereus*.

The anaerobic procedure followed differed from that of Andersen in that 12 x 300 mm. test tubes blown from lengths of 12 mm. Pyrex tubing were used, the 5% bicarbonate solution was added directly to the agar just before pouring, and the agar was poured into

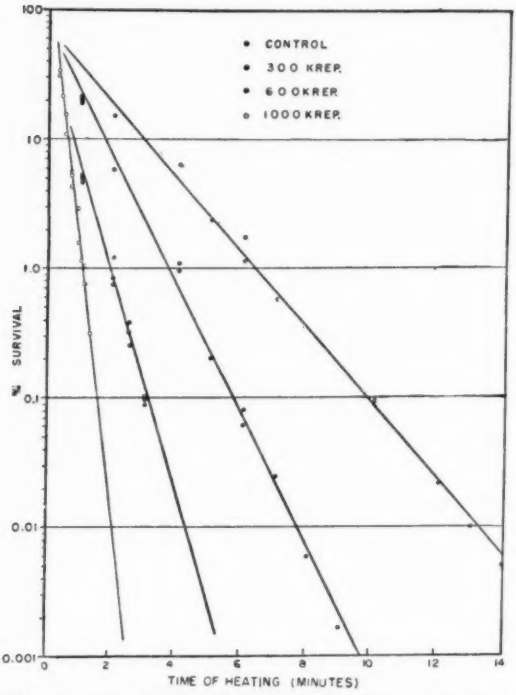

Figure 3. Effect of time of heating at 90° C. on Bacillus cereus spores that have been previously treated by 3×10^5, 6×10^5, and 1×10^6 rep of gamma radiation from a Cobalt-60 source.

the culture tubes after their inoculation with 1 ml. of diluted spore suspension. Molten vaseline stratification was used to promote anaerobiosis.

Preparation of ham medium (3.5% solids). Lean meat from a cured, but unsmoked ham was blended with sterile distilled water in a Waring blender. The ham sinews were filtered out using a cheesecloth filter and the filtrate, containing some meat tissues was sealed in polyethylene tubing and irradiated with 2.5 million rep of cathode rays for sterilization purposes. Heat-shocked bacterial suspension was mixed with the ham medium and, after irradiation by Co^{60} gamma rays, the mixture was used to inoculate the capillary sample tubes as before, except that an 18-gauge needle was used instead of a 24-gauge needle.

Ham purée experiments. Lean, cured, but uncooked, ham was chopped in a Hobart Food Cutter until it was of a gel-like consistency. A concentrated, heat-shocked spore suspension was mixed into the mass by the Food Cutter. Approximately 10 g. of this chopped, inoculated meat was spread evenly over the bottom of a 211 x 011 drawn can and the cans were sealed in polyethylene sleeves. One half of the cans were irradiated with 1×10^6 rep cathode rays from a Van de Graaff generator. All cans were then sealed. Irradiated and unirradiated samples were autoclaved at 110° C. for various periods of time. The cans were opened aseptically and the contents were blended with 99 ml. of sterile, distilled water for three minutes. Bacterial counts were made on these samples to determine the numbers of surviving organisms.

Treatment of data. Generally, 5 samples were given an equal heat treatment and irradiation as a group. Each sample of *P. A. No. 3679* spores was counted in triplicate and each sample of *B. cereus* spores in duplicate. The 15 or 10 counts thus derived

TABLE 1

Regression slopes of P.A. 3679 thermal rate of destruction curves and composite values for various doses of gamma rays

Run No.	Dose (K rep)			
	0	300	600	1000
a) *Thermal Rate of Destruction at 110° C.*				
C-9	−0.153		−0.328	
C-11	−0.127		−0.356	
C-14	−0.101		−0.354	
C-15	−0.092	−0.196		
C-16	−0.095	−0.195		
C-19	−0.135			−0.441
C-20	−0.128			−0.651
Composite Values	−0.106	−0.196	−0.354	− .595
b) *Thermal Rate of Destruction at 120° C.*				
C-23	−4.14		−8.26	
C-24	−4.54	−5.42	−7.58	
C-25	−4.22	−4.88		
C-26	−3.75	−5.63		
C-27	−4.29		−9.84	
Composite Values	−4.22	−5.22	−7.94	
c) *Thermal Rate of Destruction at 115° C.*				
C-28	−0.614			

Note: The reciprocal of the regression slope gives the time in minutes required to reduce the bacterial count by 90%.

were arithmetically averaged. Thermal rate of destruction curves were prepared by plotting the percent survival points against the duration of heat treatment at a given temperature. The mean count determined on 5 unheated samples was taken to be 100% survival and all other mean counts in the group were compared against the mean 100% value.

The best straight line for any set of points was calculated by regression analysis and was drawn through these scattered points.

RESULTS AND DISCUSSION

Figures 1 and 2 summarize graphically the effect of varying doses of gamma radiation pretreatment on the heat resistance of *P. A. No. 3679* spores at 110 and 120° C. Each curve in these figures is composed of two or more runs so that series of points may be displaced vertically to give the appearance of greater divergence than actually exists. Table 1, showing the regression slopes for each run, gives a measure of the reproducibility of the method.

It is evident that increasing doses of gamma radiation increase correspondingly the sensitivity of *P. A. No. 3679* spores to heat.

Parallel experiments with *B. cereus* are graphically summarized in Figures 3 and 4. Because of their lower heat resistance, the thermal rates of

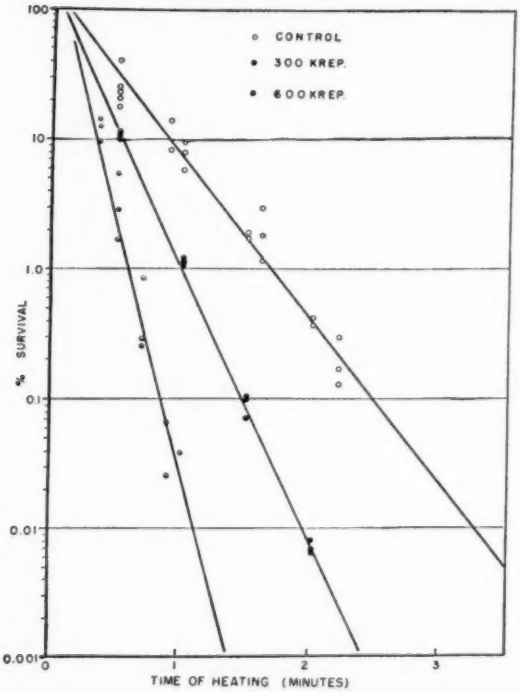

Figure 4. Effect of time of heating at 95° C. on Bacillus cereus spores that have been previously treated by 3×10^5 and 6×10^5 rep of gamma radiation from a Cobalt-60 source.

destruction curves of *B. cereus* are determined at 90 and 95° C. The thermal resistance of *B. cereus* is also reduced by doses of gamma radiation.

Another experiment was carried out to test the effect of various heat treatments on the radiosensitivity of *P. A. No. 3679* spores, the reverse of the previous experiments.

"Unpasteurized" spore suspension was sealed in capillary sample tubes and 3 groups of tubes were heated, using the Stern apparatus, for 7 minutes at

TABLE 2

Regression slopes[1] of P.A. 3679 rate of radiation destruction subsequent to three different heat treatments

Run No.	Heat Treatment		
	7 min. at 100° C.	6 min. at 110° C.	0.6 min. at 120° C.
F-2	−0.246	−0.223	−0.194
F-3	−0.203	−0.233	−0.241
F-4	−0.237	−0.244	−0.240
Composite Value	−0.238	−0.234	−0.235

[1] The regression slopes are expressed in units of "per 100 K rep to destroy 90% of the initial spore population.

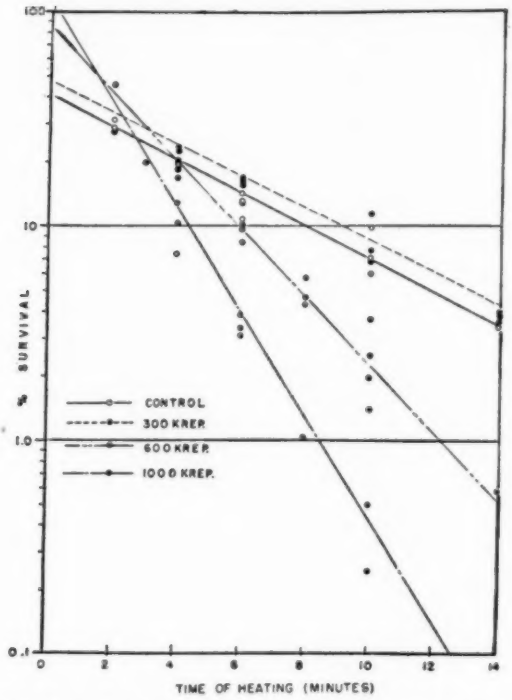

Figure 5. The effect of time of heating at 110° C. on spores of P. A. No. 3679 suspended in ham medium (3.5% solids) previously treated by 3 x 10^5, 6 x 10^5, and 1 x 10^6 rep of cathode rays.

100° C., 6 minutes at 110° C. and 0.6 minute at 120° C. These represent the usual heat shock, 90% destruction and 95% destruction levels respectively. Each group of heated samples was then irradiated with various doses of gamma rays and the survival curves were determined. The regression slope of each curve was determined and all slopes are tabulated in Table 2. It is apparent that these pre-heat treatments do not significantly affect the subsequent radiosensitivity of *P. A. No. 3679* spores.

In an attempt to approach conditions that might be encountered in actual foodstuffs, the same experiments with radiation sensitization of *P. A. No. 3679* spores to subsequent heat treatment were repeated using spores suspended in a ham medium (3.5% solids). The results of these experiments are shown in Figure 5. There is apparently a radiation protective effect exercised by the ham solids in this suspension, but at the 600 K rep dose level a radiosensitization effect is again evident.

In the final experiment *P. A. No. 3679* spores were suspended in a ham purée (22% solids) and irradiation and heat processing were carried out in metal cans. The results are presented in Figure 6. A dose of one million rep is shown to increase the heat sensitivity of *P. A. No. 3679* spores in ham

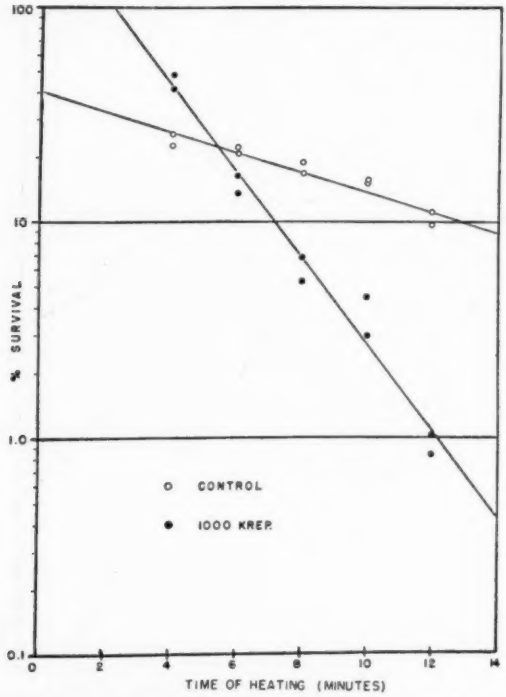

Figure 6. The effect of time of heating at 110° C. on spores of P. A. No. 3679 suspended in ham purée (22% solids) previously treated with a dose of 1×10^6 rep.

purée by a factor of four at 110° C. The results of this experiment are not strictly comparable with the results of previous experiments reported herein because, in addition to the inoculum of *P. A. No. 3679* spores, the ham also contained natural anaerobic flora of unknown properties. This experiment does demonstrate, however, that radiation sensitization of *P. A. No. 3679* spores can also occur when these spores are suspended in meat.

A plot was made (Figure 7) of the various D values obtained for irradiated and unirradiated *P. A. No. 3679* and *B. cereus* spores against the heating temperature employed. Data obtained here are not really adequate for any firm predictions to be made but were these "thermal resistance curves", or irradiated and unirradiated samples, truly parallel, it could be predicted that the radiation sensitization effect would be observable at any temperature, and that the percent reduction in D value due to a given dose of gamma rays would not be affected by the temperature of heating.

With the exception of the work by Brasch, Huber and Waly *(3)* on raw milk sterilization, most observers agree that the radiation sensitization of bacterial spores to heat is unidirectional and that heat does not sensitize spores to radiation. It is possible, but unlikely, that work with mixed flora might produce different results from work with pure cultures.

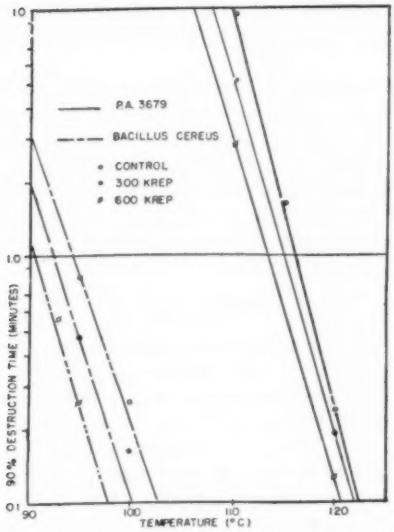

Figure 7. Plot of values for irradiated and unirradiated spores of P.A. No. 3679 and B. cereus.

Arnow *(2)*, in a review article, stated that, although heat and ultraviolet radiation induce protein coagulation in a similar manner, solubilization experiments showed that the coagulation induced by ionizing radiation is different in nature. He also stated that the general consensus was that irradiation of protein with both ultraviolet and X-rays lowered the temperature at which coagulation occurred.

Clark *(4)* found that ultraviolet radiation of salt-free albumin resulted in no change in opalescence, as measured with a Tyndall beam, until the temperature of the sample was raised to 40° C. for 1½ hours. He further suggests that a heat treatment is a necessary part of ultraviolet inactivation of bacteria, but that this is not recognized because incubation of bacteria to derive survivor counts at 37° C. would constitute sufficient heat treatment to cause ultraviolet irradiated "bacterial protein" to coagulate.

Curran and Evans *(5)* in experiments with ultraviolet irradiation of bacteria found that they were more heat sensitive after irradiation. They established a minimum radiation level below which no heat sensitization was observed. Heat applied before radiation was not as effective as that applied after irradiation, and they postulated that irradiation converts protein into some more active form. Unpublished work (1953) by the authors showed that human serum albumin irradiated with cathode rays will coagulate more rapidly on heating than will unirradiated serum albumin.

It thus appears logical to suppose that irradiation causes bacteria to become more sensitive to heat by altering some protein in their composition and making it more heat sensitive. This supposition would explain the fact that most investigators have found that prior application of heat had no effect on the radioresistance of bacteria.

No observable diminution of the sensitization effectiveness was observed even after several weeks of storage of irradiated suspensions at refrigerator temperatures.

If this effect were truly due to the ability of radiation to facilitate protein denaturation, it is possible that enzymes would show a similar sensitization effect, and furthermore, that food protein might also be made thermolabile by irradiation.

A much more detailed investigation is necessary before this process can be established as a commercial reality and it is likely that individual products will have to be studied as separate problems. The data herein do suggest, however, some interesting possibilities.

SUMMARY

The observation that doses of gamma radiation can increase the sensitivity of bacterial spores to heat has been confirmed with *Cameron's Putrefactive Anaerobe No. 3679* and *Bacillus cereus* var. *terminalis* spores. It was also found that cultures of *P. A. No. 3679* spores were similarly affected in ham menstra of 3.5% and 22% solids and that the radiosensitivity of the anaerobe is not significantly affected by two different lethal preheating treatments.

Parallels are drawn between the behavior of proteins and bacterial spores that have undergone irradiation.

Acknowledgments

The authors are grateful to Professor H. O. Halvorsön for suppplying the original culture of *Bacillus cereus* var. *terminalis*, and to Professor John T. R. Nickerson for many generous suggestions throughout the course of this work.

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THE DESTRUCTION OF THIAMINE IN MEATS AND IN AQUEOUS SOLUTION BY GAMMA RADIATION*

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Radiation studies on meats have shown that thiamine is extensively destroyed at sterilizing doses. Since radiation is a potential processing method, a survey of the thiamine destruction in a number of meats was carried out and thiamine destruction in aqueous solution was studied.

Extensive losses of thiamine have been reported for irradiated pork, beef, salmon, ham, tuna, halibut, and chicken *(1, 4)*. Thiamine in aqueous solution has been shown to be labile to radiation *(3, 7)*. Very little information is available about the mechanism of degradation of the thiamine molecule or the nature of the degradation products. It is known that little thiochrome or thiamine disulfide are among the products *(2)*.

EXPERIMENTAL

Irradiation of materials. The meats were prepared, packed, and irradiated as described previously *(4)*. Aqueous solutions of thiamine were prepared by dissolving thiamine hydrochloride in distilled water which had been boiled and cooled. The solutions were packed in No. 2 cans, gassed with nitrogen, and gamma irradiated at the Materials Testing Reactor. The samples were maintained in the frozen condition from preparation to the time of analyses except for the period during irradiation.

Methods of separation of degradation products of thiamine. Steam distillation of irradiated thiamine solution was carried out with the receiving flask at 0° C. and the vapors from this receiving flask were sparged into a second receiving flask maintained at 0° C.

Paper chromatographic separations were performed using both treated and untreated Whatman No. 1 filter paper. No differences were observed in the efficiency of separation between the untreated paper and the paper which was washed with 0.2% tetrasodium ethylenediaminetetracetate at pH 8.5; however, the resolved components were easier to locate in ultraviolet light if treated paper was used. Chromatograms were developed in a descending manner using a butanol: acetic acid: water (50:19:50) solvent.

Qualitative methods used to examine irradiated thiamine solutions. Tests for hydrogen sulfide and carbon dioxide were made by flushing the irradiated thiamine solution with nitrogen and bubbling the gas through lead acetate. The test for carbon monoxide was made in a like manner except the gas was bubbled through cuprous chloride. Ammonia was identified by its odor, its volatile basic character, and precipitation with sodium cobaltinitrate *(5)*. Thiamine was identified on the paper chromatograms by obtaining the characteristic fluorescent thiochrome after spraying with an alkaline ferricyanide solution. Infrared absorption spectra of the thiamine and irradiated thiamine were obtained using a Beckman model IR infrared spectrophotometer. Samples were prepared for the sodium chloride cells by suspending the freeze-died thiamine in mineral oil.

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* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

Quantitative methods used in studying irradiated meats and thiamine solution. Quantitative determinations for thiamine in meat and in irradiated thiamine solutions were made using the fluorimetric method as described previously *(4)*. Determinations of ammonia and any other volatile base were made by steam distilling the ammonia from an alkaline solution, pH 8, into buffer and titrating with standard acid. Pyrimidine ring destruction was measured using the absorbance at 264 mμ. The formation of the yellow-colored substance was measured using the absorbance at 494 mμ. The visible spectra of the irradiated thiamine solution showed little character, however, there was a shoulder at 494 mμ. The fluorescence of the irradiated solution was measured using a photofluorimeter and quinine sulfate as a standard.

RESULTS AND DISCUSSION

Destruction of thiamine in meats. The retention of thiamine in irradiated lamb, veal, bacon, lake trout and turkey is given in Table 1. These data given here are the results of an extension of a previous study *(4)* and these findings agree with those reported by Alexander *(1)*. The loss of thiamine in irradiated meat is an undesirable nutritional change and is the greatest nutrient change in irradiated meats recorded to date.

TABLE 1

Retention of thiamine in lamb, veal, lake trout, and turkey irradiated in a nitrogen atmosphere

Dose	Thiamine retention in				
	Lamb 0.12 mg./100 g.[1]	Veal 0.09 mg./100 g.[1]	Bacon 0.37 mg./100 g.[1]	Lake Trout 0.11 mg./100 g.[1]	Turkey 0.14 mg./100 g.[1]
rep x 10⁻⁶	%	%	%	%	%
0.5	58	56	60	36	43
1.0				18	
1.3					21
1.4	33	44	41		
2.0	25	22	35	9	14
4.0	17	11	16	9	7

[1] Thiamine content of unirradiated control.

Destruction of thiamine in aqueous solution. The destruction of thiamine in aqueous solution is a function of radiation dose. Irradiated thiamine solution is yellow in color, fluorescent, and has an odor similar to burned rubber and thiazole. During irradiation the pH of the solution is lowered and also ammonia is formed. Qualitative tests for hydrogen sulfide, carbon monoxide, and carbon dioxide were negative. The fluorescent and yellow-colored substances were found to be insoluble in diethyl ether, benzene, and petroleum ether. Treatment of the solution with alkali increased the intensity of the yellow color. This yellow color might be due to the formation of a compound similar to the known thiazole dyes which have structures of condensed thiazoles and yellow colors. The residue of the lyophilized, irradiated thiamine solution had a yellow-brown color and was soluble in water and ethanol. The quantitative changes measured in irradiated thiamine solution are given in Table 2.

The most interesting differences between the infrared spectra of irradiated thiamine and thiamine were the decreases in absorbance at 6.0 and 6.2 μ shown in Figure 1. This doublet absorbance at 6.0 and 6.2 μ in thiamine has

TABLE 2

The effects of radiation on aqueous thiamine

Dose	Thiamine[1] destruction	Pyrimidine ring destruction	Ammonia formation	Acid formation	Yellow color formation	Fluorescence[2] formation
rep x 10⁻⁶	*mM*	*mM*	*mM*	*mM*	*absorbance*	*mg./L.*
2	8.0	2.1	1.0	3.9	0.025	0.4
10	17.5	5.5	7.0	8.0	0.173	2.6
20	26.3	10.4	.....	12.8	.....	16.5
40	28.1	16.6	21.2	15.6	0.271	42.4

[1] Original solution contained 28.8 mM thiamine.
[2] In terms of quinine sulfate.

been assigned to the amino-pyrimidine *(6)* and is probably caused by C = N stretching and N-H bending, respectively *(8)*. These decreases in absorbance correlate with the other results and indicate destruction of the pyrimidine ring and the loss of the amino group.

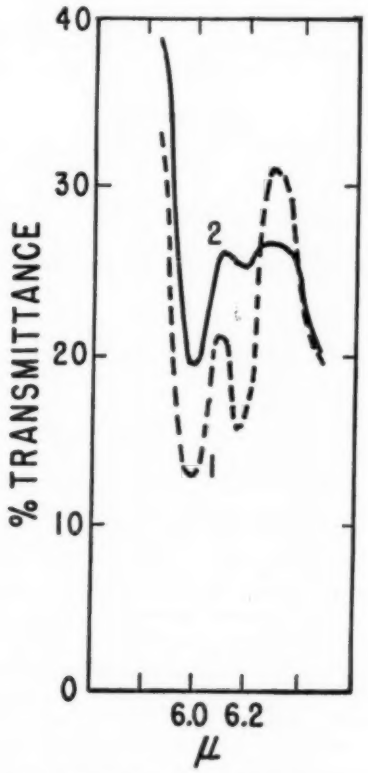

Figure 1. Changes in infrared spectra.
1. Thiamine
2. Irradiated thiamine, 10 x 10⁶ rep.

Aqueous thiamine solution, irradiated at doses above 10 x 10^6 rep, was separated by paper chromatography into 3 components. One component with an Rf of 0.43 was ninhydrin positive and was present at all radiation levels as shown in Figure 2. Approximately the same amounts were formed at doses of 10, 20 and 40 x 10^6 rep; however, somewhat smaller amounts were formed at 2 x 10^6 rep. A second component with an Rf of 0.62 was highly fluorescent under ultraviolet light. This component was not observed at 2 x 10^6 rep, but was presented in increasing amounts at higher doses. The third component with an Rf of 0.55 was thiamine. Attempts to identify the eluted components by their absorption spectra were unsuccessful. The ninhydrin-positive component of the chromatogram is probably the yellow-colored substance of the solution and the fluorescent component is probably the fluorescent substance found in the solution.

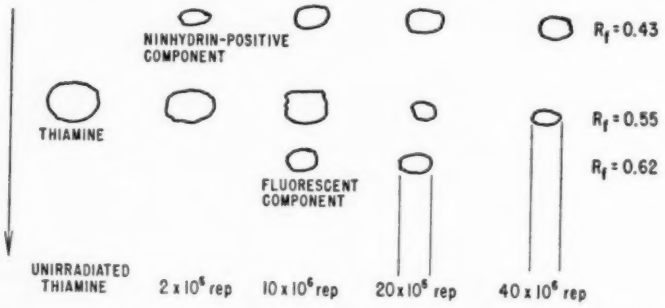

Figure 2. Reproduction of paper chromatograms showing thiamine degradation products.

The formation of the yellow-colored substance in irradiated thiamine solution decreased at increasing doses. This would indicate that the yellow-colored substance was utilized in a secondary reaction. The fluorescent substance in irradiated thiamine solution was formed in small amounts at doses to 10 x 10^6 rep; however, above this dosage the fluorescent substance increased as a function of dose. This would indicate that the fluorescent substance was formed by a secondary reaction from a primary breakdown product of thiamine. Schopfer *(10)* has reported that ultraviolet irradiation of thiazole will form a fluorescent product, which he called flurochrome. Attempts to separate the yellow-colored substance and the fluorescent substance from the unreacted thiamine by steam distillation techniques and by solubility differences were unsuccessful.

Ammonia was formed in irradiated thiamine solution as a function of radiation dose indicating that this is a primary reaction. The source of the ammonia is probably the 6-amino group of the pyrimidine portion of thiamine; it is less likely to come from the thiazole and the pyrimidine rings. The possibility that the ammonia originates from the pyrimidine ring nitrogen is considered less likely because there is little correlation between the formation of ammonia and the destruction of pyrimidine (Table 2).

The formation of acid decreases with increased dose indicating that the acid products participate in secondary reactions or are neutralized by the formation of a basic substance.

Pyrimidine ring destruction decreased with increasing dose. The breaking of the pyrimidine ring accounted for 26% of the thiamine destroyed at 2×10^6 rep and 59% at 40×10^6 rep. Scholes *(9)* has reported that the pyrimidine ring of nucleic acids is broken by ionizing radiation.

SUMMARY

It has been shown that thiamine is destroyed in meats and in aqueous solution by gamma irradiation. Irradiated aqueous thiamine solution is yellow-colored, fluorescent and has an odor like that of burned rubber or thiazole. There is a formation of ammonia and titratable acid and a destruction of pyrimidine ring. It appears that the primary reaction occurring during the irradiation of thiamine is the formation of ammonia. The source of the ammonia is probably the 6-amino group of the pyrimidine moiety, but it could also come from the thiazole and pyrimidine ring nitrogen. Paper chromatographic separation indicated that two main degradation products are formed during irradiation. Attempts to characterize these products using their absorbance spectra have been unsuccessful.

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FLAVOR IN FOODS—A CUMULATIVE REVIEW[a]

Recent Contributions to the Chemistry of Flavor in Milk and its Products[b]

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Milk and its products are accepted by the consumer primarily on the basis of flavor. Flavor in dairy foods ranges from the faint (milk) to the intense (cheese). Thus, flavor objectives vary among dairy products and in general flavor control is concerned with (a) prevention of off-flavor, (b) maintenance of desired flavors, and (c) development of characteristic flavors. In recent years, better control of flavor problems has been sought through fundamental investigations of the chemical identity, origin, and mode of formation of flavor compounds. This paper attempts to give a brief resume of recent findings in this area of research with reference to the principal dairy products. It does not represent a complete literature review, and the subject of added flavors, which are particularly important in ice cream, is excluded.

FLUID MILK

In connection with milk flavor, methyl sulfide is of much interest. It has a malty-cowy aroma at proper dilution and has been revealed in raw milk *(13)*. Other compounds contributing to the normal flavor of milk await identification. Feed flavors which gain entry to milk through the cow, represent the most important off-flavors of market milk. In order to remove such flavors from milk "vacuumizing" treatment is coming into common practice. However, there have been no recent studies regarding identity of compounds responsible for feed-type flavors.

Three other important flavor defects of market milk are those termed "oxidized", "rancid", and "sunlight". The compounds responsible for oxidized flavor in skimmilk recently have been identified by Forss *et al. (2)* as a series of unsaturated aldehydes. The normal alkyl 2-enals containing 5 to 11 carbons inclusive as well as several dienals were implicated in the flavor. 2-Octenal and 2-nonenal were observed to exhibit the flavor most typically. These compounds arise through autoxidation of the milk lipids.

Rancid flavor, due to hydrolytic splitting of milk fat, has always been a problem in milk since milk contains lipase which may be easily activated in a number of ways. It has been known for many years that the lower fatty acids, particularly butyric acid, of milk fat are responsible for this flavor. Current research efforts are directed toward characterization of the milk lipases,

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[a] This feature of FOOD RESEARCH was initiated in the January-February issue, 1957, continued in the March-April issue, and thereupon, due to a change in plan recommended by Dr. George W. Kurtz, Qm. Food and Container Institute for the Armed Forces, Chicago, Illinois, was suspended until this issue. Dr. Kurtz has kindly consented to coordinate the series. Ed.

[b] Authorized for publication as paper No. 2181 on July 30, 1957 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

determining their mode of action and developing preventive methods in milk processing *(4, 14, 15)*.

On exposure to light, especially strong sunlight, milk develops objectionable flavors. There are two aspects to this defect, one being the oxidized flavor and the other deriving uniquely from the effects of light. Patton *(10)* has presented evidence that sunlight flavor is due to methional (3-methylmercaptopropionaldehyde) which results from the photolytic decomposition of methionine in the presence of riboflavin. Methional may be of general importance in the flavor of foods because of its broth-like qualities and its ease of formation from methionine during cooking *(12)*. However, Witting and Batzer *(16)* have questioned whether it has any odor at all. They claim that methional when freshly vacuum distilled is odorless and that within minutes after exposure to air it takes on the extremely potent meat-like character. They further propose that this odor results from methional sulfoxide. The observations of Witting and Batzer concerning methional could not be confirmed at this laboratory.

Another characteristic off-flavor of fluid milk is that termed "malty". Jackson and Morgan *(5)* have established that isovaleraldehyde (3-methyl butanal) resulting from the action of certain species of Streptococcus on the amino acid leucine is responsible for this flavor. This defect earlier had been attributed to acetaldehyde [see literature review, *(5)*].

Much interest exists currently in radiation preservation of foods. One of the principal obstacles to this application is off-flavor development. Some recent observations by Day *et al. (1)* on the effects of gamma radiation of skimmilk are of interest. The carbonyl compounds resulting from the exposure of such milk to a dosage of 2×10^6 rep were found to include acetaldehyde, acetone, butanone, formaldehyde, and n-hexanal. Of these acetaldehyde was of principal flavor importance. The primary off-flavor in these studies resulted from low molecular weight, reduced sulfur compounds. Using gas chromatography and mass spectrometry, methyl mercaptan and methyl sulfide were shown in radiated milk and radiated simplified systems thereof.

Another flavor of milk involving reduced sulfur is cooked flavor. Heat denaturation of the milk serum proteins, particularly β-lactoglobulin, and proteins of the milk fat globule membrane produces volatile sulfides, largely H_2S. These sulfides first become evident in milk organoleptically after momentary heating to approximately 170° F. The mechanism of H_2S formation in this instance has not been satisfactorily elaborated *(11)*.

In general flavor findings on whole milk also are somewhat applicable to cream and skimmilk.

CONCENTRATED MILKS

The two concentrated milk products of primary interest from a flavor standpoint are evaporated milk and dry milks. Although many consumers like the flavor of evaporated milk for various purposes, it is considered that the product would have greater flexibility if it could be used extensively for beverage purposes. The heat treatment (retort process, about 240° F. for 15 minutes) employed in sterilizing this product changes the flavor rather drastically. Use of so-called short time-high temperature sterilizing pro-

cedures coupled with aseptic packing minimize, but do not prevent, these flavor changes. A host of compounds contribute to the flavor of evaporated milk and perhaps the best overall descriptive term is "caramelized". Morgan *et al. (9)* have reported that the principal volatile carbonyls of sterilized milks are acetaldehyde and furfuraldehyde. Keeney and Patton *(7)* have shown that evaporated milk contains δ-decalactone, a compound possessed of a heated butter-type flavor (see below). Methyl sulfide and a group of methyl ketones, including the 3, 5, and 7 carbon members, have been revealed by Wong *et al. (17)* in evaporated milk. These ketones are produced through the effects of moist heat on milk fat. The theory that they result from hydrolytic cleavage and decarboxylation of beta-ketoacids (unreported) in milk fat is attractive. It seems improbable that these few compounds can adequately account for the total flavor of evaporated milk. It has been pointed out elsewhere *(11)* that the caramelized flavor in this product results from the interaction of lactose with the milk proteins. However, identification of the caramel compounds has not been accomplished.

Research efforts to develop a suitable beverage grade dry whole milk have been conducted continuously since the early 1940's. This problem has two facets of importance, one being flavor and the other dispersibility. Although oxidative deterioration of dry whole milk is now relatively well controlled through processing and inert gas packing, certain other spontaneous changes in flavor limit utility of the product. One of these concerns the formation of δ-decalactone which originates in the lipid phase of the product *(7)*. Although the buttery, coconut-like flavor character of this compound is not unpleasant, it is alien to beverage milk and thus objectionable. This compound appears to be formed in a non-oxidative mechanism and a reasonable origin in view of present knowledge is 5-hydroxydecanoic acid. The precursor of the lactone tends to concentrate itself in the low melting portion of the milk fat.

As storage of dry whole milk progresses a flavor termed "stale" develops. The precise chemical character of this flavor is unknown and it probably represents the principal barrier to development of a product with acceptable flavor and long shelflife at room temperature. Undoubtedly, the browning reaction (early uncolored stage) is partially responsible for this flavor.

In general, the findings concerning dry whole milk hold at least in part for dried cream, dried ice cream mix, dried buttermilk, and nonfat dry milk. Where air packing is employed for such products oxidized flavor also becomes a problem.

BUTTER

Butter can be differentiated on the basis of the raw material employed. Uncultured sweet cream gives a product of much milder flavor than does cultured cream. Butter made from the latter is offered more extensively on the continent whereas sweet cream butter is more widely consumed in the United States. Beyond study of the factors governing diacetyl formation, which phenomenon has been known for many years, no new investigations of flavor compounds in butter from either cultured or uncultured cream have been reported. The flavor of butter is most attractive and would seem to be a worthy object of further investigation. As mentioned above δ-decalactone is known to be formed when milk fat is heated and this compound no doubt

accounts for some of the pleasant aroma developed when foods are cooked or baked with butter. It is of interest in this connection that patents (Brit. 748,645; 748,661; 748,801) have been granted covering the preparation and use of certain aliphatic lactones for flavoring margarine.

CHEESE

The chemical identity of cheese flavor, particularly that of cheddar cheese, has presented a strong challenge to research workers for many years. The problem is complex and far from satisfactorily solved. It is common knowledge that the lower fatty acids contribute to the flavor of most cheeses. However, various mixtures of these prove inadequate in simulation of the flavor. Methyl ketones are known to render the flavor of mold-ripened cheeses distinctive from that of other varieties and recently Morgan and Anderson *(8)* have confirmed the presence of the odd numbered methyl ketones from C_3 to C_{11} in such cheese. The most characteristic of these so far as blue cheese flavor is concerned is methyl amyl ketone (heptanone-2).

Keeney and Day *(6)* have recently proposed that methional (see above) is an important flavor contributor to cheddar cheese. Observations at the authors' laboratory have shown that the Strecker degradation of leucine, contaminated with a small amount of methionine produces a "toasted" cheddar aroma. The two principal compounds which should result under such conditions are isovaleraldehyde and methional. In any event, it is clear that no single compound accounts for the full characteristic flavor of any given variety of cheese. A recent review of the biochemistry of cheese ripening by Harper and Kristoffersen *(3)* points out the numerous possible routes of flavor formation.

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